

THE ROLE OF ANTIBODY IN CELL-MEDIATED IMMUNITY TO
NONTYPHOIDAL *SALMONELLA* IN AFRICAN CHILDREN
AND HIV-INFECTED ADULTS

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A thesis submitted to the
University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

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September 2012

ABSTRACT

Nontyphoidal *Salmonella* (NTS) are a major cause of invasive disease in young children and HIV-infected adults in Sub-Saharan Africa. To develop a vaccine for NTS, an improved understanding of immunity to NTS is required. This thesis investigates the role of opsonic antibody in protection against NTS. First, we defined the optimal serum handling conditions to preserve complement function. We determined minimal titres of antibody and complement required for cell-mediated killing of *Salmonella* and found they are higher than for cell-free killing. We demonstrated impaired blood cell killing of NTS opsonised with sera from HIV-infected Africans. Developing a method to purify anti-LPS antibodies, we showed that high titres of anti-LPS antibodies in these sera inhibit cell-mediated killing while lower titres are opsonic and induce cell-mediated killing of NTS. For most children, antibody acquired during NTS bacteraemia effected cell-mediated killing of NTS. High antibody titres were not necessarily protective, but for some sera, dilution prior to opsonisation, induced killing. The sensitivity of Malawian NTS isolates to opsonic antibody varied, with resistance to cell-mediated and bactericidal killing correlating. Overall, this thesis emphasises the importance of opsonic antibody in protecting against NTS and supports the development of a vaccine which induces antibody to *Salmonella*.

DEDICATION

I would like to dedicate this thesis to my wonderful family and friends because without their love and support none of this would have been possible.

ACKNOWLEDGEMENT

First of all, thank you to my supervisor Professor Cal MacLennan. It has been quite a journey and I have been extremely fortunate to share it with such a supportive, caring, patient and encouraging supervisor. I would not have wanted to do it without you. Thank you for continuing to inspire me and for starting me on the research path. I will be forever indebted. I am also grateful to my co-supervisor Professor Adam Cunningham for his advice, reassurance and continued support.

Many thanks to everyone in our lab – John, for always being positive, your unfailing help and wisdom, and for making a million agar plates! To Jenny, for being so caring and sharing your experience, and of course Pete – thank you for being there for me throughout the highs and lows of my PhD. I cherish many happy memories of the past 2 years and hope there are more to come. I would like to express my thanks also to the ‘fourth floor girls’ in the IBR. Your love, laughter and friendship over the past 3 years has been incredible.

I would like to thank everyone at Novartis Vaccines Institute for Global Health, Italy for making me so welcome and my time in Siena so enjoyable and productive, especially the post-docs Yunshan Goh, Francesca Necchi and Simona Rondini. You all taught me so much. Very special thanks to Francesca Micoli for your enthusiasm and patience and for never giving up with the antibody purification. We got there in the end!

I acknowledge my appreciation to the Medical Research Council for financial support, and to everyone at the Malawi-Liverpool-Wellcome Trust facility, Malawi who collected and processed the samples that enabled me to carry out this research.

Last but not least, thank you to Madre, Padre, Frances and Craig for their never ending love and support. I could not have done it without you.

CONTENTS

CHAPTER 1.

INTRODUCTION	1
1.1 Theme and overview	1
1.2.1 <i>Salmonella</i>	1
1.2.2 Taxonomy	2
1.2.3 <i>Salmonellae</i> as pathogens	2
1.3 Epidemiology of NTS infections	3
1.3.1 <i>Salmonella</i> infections in developed countries	3
1.3.2 <i>Salmonella</i> infections in Africa	3
1.3.3 Clinical presentation	4
1.3.4 Treatment	6
1.4 Risk factors for invasive nontyphoidal <i>Salmonella</i> disease in Africa	6
1.4.1 Environmental risk factors	8
1.4.2 Young age	8
1.4.3 Malaria and malarial anaemia	8
1.4.4 HIV	9
1.4.5 Malnutrition	10
1.5 <i>Salmonella</i> pathogenesis	11
1.6 <i>Salmonella</i> virulence and resistance to killing	14
1.6.1 <i>Salmonella</i> pathogenicity islands	15
1.6.2 LPS	15
1.6.3 PhoP/Q	16
1.7 Immunity to <i>Salmonella</i>	16
1.7.1 Phagocytes	17
1.7.2 Cytokines	20
1.7.2.1 TNF- α	20
1.7.2.3 IL-12	21

1.7.2.4 IL-15	22
1.7.2.5 IL-18	22
1.7.2.6 IL-10 and IL-4	23
1.7.3 T cells	23
1.7.4 B cells	24
1.7.5 Complement	25
1.7.6 Antibody	27
1.8 Vaccines against <i>Salmonella</i>	30
1.9 Aims and objectives	32

CHAPTER 2.

MATERIALS AND METHODS 34

2.1 Ethical approval	34
2.2 Laboratory methods	34
2.2.1 Bacteria	34
2.2.1.2 Preparation of <i>Salmonella</i> enterica serovar Typhimurium stationary phase culture	34
2.2.1.3 Preparation of <i>Salmonella</i> Typhimurium in logarithmic phase	36
2.2.2 Preparation of human serum	36
2.2.3 Serum Bactericidal Assay (SBA)	37
2.2.4 Preparation of washed blood cells	37
2.2.5 Intracellular blood cell killing assay	Error! Bookmark not defined.
2.2.6 Quantification of phagocytic activity of neutrophils and monocytes in washed blood in response to stimulation with <i>Salmonella</i>	39
2.2.6.1 Labelling of <i>Salmonellae</i> with FITC	39
2.2.6.2 Phagocytosis assay	40
2.2.7 Quantification of oxidative burst activity of neutrophils and monocytes in washed blood in response to stimulation with <i>Salmonella</i> Typhimurium	41
2.2.8 Enzyme-linked immunosorbent assays (ELISA) to quantify relative amount of anti-LPS antibodies in serum and eluates	43

2.2.9 Flow cytometric antibody assay to quantify binding of antibody and complement to the surface of <i>Salmonellae</i>	46
2.2.10 Haemolytic assays	48
2.2.11 Absorption of anti-LPS antibodies from human serum using LPS attached to a solid matrix	50
2.2.12 Depletion of anti- <i>Salmonella</i> Typhimurium D23580 antibodies from human serum	50
2.2.13 Affinity purification of anti-O-Antigen antibody from human serum using activated O-Antigen linked to NHS-Sepharose resin	52
2.2.13.1 Preparation of O-Antigen from LPS	52
2.2.13.2 Phenol sulphuric assay	52
2.2.13.3 Activation of O-Antigen with ADH	53
2.2.13.4 Activation of O-Antigen with ADH to produce OAgADH	53
2.2.13.5 Activation of O-Antigen with ADH to produce OAgoxADH	54
2.2.13.6 Immobilization of activated O-Antigen to the resin of HiTrap NHS columns	54
2.2.13.7 Ammonium sulphate precipitation of serum proteins	55
2.2.13.8 Affinity purification of anti- <i>S. Typhimurium</i> O-Antigen antibodies from human serum	56

CHAPTER 3.

STABILITY OF COMPLEMENT-MEDIATED BACTERICIDAL ACTIVITY IN HUMAN SERUM AGAINST NONTYPHOIDAL SALMONELLA

3.1 Introduction	57
3.2 Objectives	59
3.3 Results	59
3.3.1 Human sera used in the study contained <i>Salmonella</i> -specific antibody	59
3.3.2 Serum bactericidal activity against NTS is retained for at least 35 days when sera is stored at 4°C and three freeze-thaw cycles do not have an effect on complement stability	60

3.3.3 Classical and alternative pathway haemolytic complement activity is reduced over time	62
3.3.4 Serum has reduced longevity of bactericidal activity against NTS when stored at 22°C and 37°C	64
3.3.5 The percentage of human serum required to kill NTS increases incrementally with time	64
3.3.6 Delayed separation of serum from clotted human blood for up to four days post-venesection does not reduce ability to kill NTS	67
3.4 Discussion	70
3.5 Conclusions	74

CHAPTER 4.

IDENTIFICATION OF AN EXOGENOUS COMPLEMENT SOURCE FOR KILLING OF <i>S. TYPHIMURIUM</i> BY BLOOD CELLS	76
4.1 Introduction	76
4.2 Objectives	77
4.3 Results	78
4.3.1 Opsonic activity of diluted human serum is reduced compared to whole serum	78
4.3.2 BRS does not contain antibodies to <i>S. Typhimurium</i> D23580	80
4.3.3 BRS is toxic to <i>S. Typhimurium</i> D23580 at high concentrations	80
4.3.4 Functional human complement remains in serum after pre-absorption of anti- <i>S. Typhimurium</i> D23580 antibodies	84
4.3.5 Antibody-depleted human serum is a suitable complement source for blood cell killing of <i>S. Typhimurium</i> D23580	86
4.3.6 Complement and antibody requirement for serum bactericidal killing of <i>S. Typhimurium</i> D23580 is lower than that for blood cell killing	88
4.4 Discussion	90
4.5 Conclusions	93

CHAPTER 5.

IMPAIRED CELL-MEDIATED IMMUNITY TO NTS IN HIV-INFECTED AFRICAN ADULTS

	95
5.1 Introduction	95
5.2 Objectives	97
5.3 Results	97
5.3.1 Killing of <i>S. Typhimurium</i> D23580 by peripheral blood cells is impaired when opsonised with serum from a subset of HIV-infected African adults	97
5.3.2. Phagocytosis of <i>S. Typhimurium</i> D23580 opsonised with serum from some HIV-infected African adults is impaired	102
5.3.3 Oxidative burst by blood phagocytes is not reduced in response to stimulation with <i>S. Typhimurium</i> opsonised with HIV-infected or HIV-uninfected adult sera	104
5.3.4 Bactericidal killing of <i>Salmonellae</i> does not occur during the opsonisation period	106
5.3.5 All sera can deposit C3 on <i>Salmonella</i>	107
5.3.6 HIV-infected inhibitory serum can kill <i>S. Typhimurium</i> D23580 when diluted with HIV-uninfected serum	107
5.3.7 HIV-infected inhibitory sera have higher serum anti-LPS IgG and IgA titres	Error!
Bookmark not defined.	
5.3.8 <i>S. Typhimurium</i> mutant <i>galE</i> - opsonised with serum from Malawian adults is killed by peripheral blood cells	112
5.3.9 Removal of LPS antibodies restores killing of <i>Salmonella</i> opsonised with HIV-infected inhibitory serum	114
5.3.10 Removal of antibodies to LPS from different serovars or components of LPS <i>S. Typhimurium</i> does not restore killing of <i>S. Typhimurium</i> D23580 by washed blood cells	116
5.3.11 High concentrations of purified O-antigen antibodies inhibit cell-mediated killing of <i>S. Typhimurium</i> D23580	117
5.3.12 Purified anti- <i>Salmonella Typhimurium</i> O-antigen antibodies have an opsonic function	121

5.4 Discussion	125
5.5 Conclusions	132

CHAPTER 6.

PURIFICATION OF ANTIBODIES TO O-ANTIGEN OF *S. TYPHIMURIUM* FROM HUMAN SERUM BY AFFINITY CHROMATOGRAPHY

134

6.1 Introduction	134
6.2 Objectives	136
6.3 Results	137
6.3.1 Purification of O-Antigen	137
6.3.2 Activation of purified O-Antigen with ADH and coupling to NHS-Sepharose column	139
6.3.3 Testing antigenic integrity of activated O-Antigen	140
6.3.4 Purification of antibodies from human serum	142
6.3.5 Optimising binding of antibodies to OAgADH-NHS-Sepharose columns	146
6.3.6 Comparing elution of antibodies from OAgADH column with glycine at four different pH as the elution buffer	149
6.3.7 Comparing elution of antibodies from OAgADH column with four different elution buffers	150
6.3.8 Sequential elution of anti-O-Antigen antibodies	152
6.3.9 Decreasing the amount of activated O-Antigen coupled to NHS-Sepharose increases the recovery of purified antibody	154
6.3.10 Purification of anti-O-antigen antibodies from HIV-infected Malawian adult serum	158
6.4 Discussion	160
6.5 Conclusions	163

CHAPTER 7.

OPSONIC ANTIBODY AND IMMUNITY TO NTS IN MALAWIAN CHILDREN WITH BACTERAEMIA **165**

7.1 Introduction	165
7.2 Objectives	166
7.3 Results	166
7.3.1 Study participants	166
7.3.2 <i>Salmonellae</i> opsonised with acute and convalescent sera from some children could not be killed by peripheral washed adult blood cells	167
7.3.3 Effect of age on cell-mediated immunity to NTS in bacteraemic children	169
7.3.4 HIV infection and bacteraemic children	171
7.3.5 Anti- <i>Salmonellae</i> antibody titres and blood-cell killing of <i>Salmonellae</i> opsonised with acute and convalescent children's sera	173
7.3.6 Anti- <i>Salmonella</i> antibody levels increase after infection	177
7.3.7 Blood cell killing of <i>Salmonella</i> is enhanced when opsonised with dilutions of acute and convalescent sera	177
7.3.8 Killing of <i>Salmonella</i> opsonised with diluted antibody by peripheral blood cells compared with Anti- <i>Salmonella</i> antibody levels	182
7.4 Discussion	182
7.5 Conclusions	187

CHAPTER 8.

SENSITIVITY OF MALAWIAN *SALMONELLA* ISOLATES TO KILLING BY ANTIBODY **189**

8.1 Introduction	189
8.2 Objectives	190
8.3 Results	190
8.3.1 Serovar distribution and case fatality rates of NTS bacteraemia	190
8.3.2 Sensitivity of Malawian NTS isolates to killing by antibody	190
8.3.3 Sensitivity of Malawian NTS isolates to opsonic antibody and killing by peripheral blood cells	191

8.3.4 Sensitivity of isolates to phagocytosis and oxidative burst	193
8.3.5 Antibody and complement deposition on Malawian isolates	196
8.4 Discussion	199
8.5 Conclusions	202
 CHAPTER 9.	
GENERAL DISCUSSION	203
 CHAPTER 10: REFERENCES	209
 APPENDIX A	224
APPENDIX B	234

LIST OF FIGURES

1.1	Map of Africa highlighting blood stream infection studies	5
1.2	Clinical presentation of invasive NTS disease in African children and adults	7
1.3	Distribution of <i>Salmonella</i> in the mouse model	12
1.4	Phases of primary <i>Salmonella</i> infection	18
1.5	Activation and function of the three complement pathways	26
1.6	The key immunological factors that control the intracellular and extracellular phases of infection in humans	28
2.1	Serum bactericidal assay analysis	38
2.2	Identification of neutrophil and monocyte populations in peripheral blood with flow cytometry	42
2.3	Dilution of serum in a 96 well ELISA plate	45
2.4	Identification of <i>Salmonellae</i> population with a flow cytometer	47
2.5	Radial immunodiffusion assay to measure haemolytic complement activity	49
2.6	Anti-LPS antibody depletion of human serum	51
3.1	Levels of anti- <i>S. Typhimurium</i> D23580 IgG, IgA and IgM antibodies in human sera used in the study as assessed by flow cytometry	61
3.2	Effect of duration of storage of human serum at 4°C and freeze-thaw cycles on its ability to kill <i>S. Typhimurium</i> D23580	63
3.3	Classical and alternative pathway haemolytic complement activity in human serum stored at 4°C for 91 days	65
3.4	Effect of duration of storage on human serum at 22°C and 37°C on ability to kill <i>S. Typhimurium</i> D23580	66
3.5	Percentage of human serum stored at 22°C required to kill <i>S. Typhimurium</i> D23580	68
3.6	Effect of delayed separation of serum from clotted blood on ability to kill <i>S. Typhimurium</i> D23580	69
4.1	Blood cell killing of <i>S. Typhimurium</i> D23580 opsonised with diluted human	79

	serum	
4.2	Anti- <i>Salmonella</i> Typhimurium D23580 antibody levels in BRS and human serum	81
4.3	Killing of <i>S. Typhimurium</i> D23580 opsonised with BRS alone or supplemented with antibody	82
4.4	Antibody-depleted human serum contains no antibody to <i>Salmonella</i> but complement remains functional	85
4.5	Antibody-depleted serum and antibody requirement for blood cell killing of <i>S. Typhimurium</i> D23580	87
4.6	Requirement of antibody and complement for bactericidal killing of <i>S. Typhimurium</i>	89
5.1	Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on killing of <i>Salmonellae</i> by adult washed peripheral blood cells	100
5.2	Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on phagocytosis of <i>Salmonellae</i> by adult washed neutrophils and monocytes	103
5.3	Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on oxidative burst of adult washed neutrophils and monocytes	105
5.4	No bactericidal killing of <i>S. Typhimurium</i> D23580 occurs during the 20 min opsonisation period	108
5.5	Inhibition of opsonic killing of <i>S. Typhimurium</i> by HIV-uninfected serum mixed with HIV-infected inhibitory serum	110
5.6	HIV-infected inhibitory sera have higher anti- <i>S. Typhimurium</i> LPS IgG and IgA titres	111
5.7	Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on killing of <i>Salmonella</i> Typhimurium <i>galE</i> - mutant which has a truncated OAg chain by adult washed peripheral blood cells	113
5.8	Effect of opsonising <i>S. Typhimurium</i> D23580 with HIV-uninfected or HIV-	115

	infected sera preabsorbed with <i>S. Typhimurium</i> LPS	
5.9	Effect of pre-absorbing HIV-infected sera with different components of <i>Salmonella</i> LPS and LPS from <i>S. Enteritidis</i> and <i>S. Minnesota</i> on blood cell killing of <i>S. Typhimurium</i> D23580	118
5.10	Effect of anti-OAg antibodies on killing of <i>S. Typhimurium</i> D23580 by washed blood cells	120
5.11	Effect of anti-OAg antibodies on killing of <i>S. Typhimurium</i> D23580 by washed blood cells	122
5.12	Blood cell killing of <i>S. Typhimurium</i> D23580 opsonised with human complement and diluted purified anti-OAg antibodies	124
6.1	Structure of <i>S. Typhimurium</i> LPS	138
6.2	Reaction schemes for production of activated OAg	141
6.3	Activated OAg from <i>S. Typhimurium</i> D23580 coupled to NHS-Sepharose can reversibly bind antibody	143
6.4	Activated OAg from <i>S. Typhimurium</i> D23580 coupled to NHS-Sepharose can reversibly bind human polyclonal antibody	145
6.5	Decreased binding of anti-OAg antibodies from non-precipitated human serum or from Vivaspin treated human serum preparation	147
6.6	Elution of anti-OAg antibodies from human serum using glycine with a decreasing pH	151
6.7	Ability of different buffers to elute bound anti-OAg IgG antibodies from human or rabbit serum	153
6.8	Sequential elution of antibody	155
6.9	Variation in amount of activated OAg immobilised to NHS-Sepharose affects recovery of bound antibody from affinity columns	157
6.10	Purification of IgG, IgA and IgM antibodies to OAg of <i>S. Typhimurium</i> from a HIV-infected African adult serum	159
7.1	Blood cell killing of <i>S. Typhimurium</i> D23580 opsonised with acute and convalescent sera	168
7.2	No correlation between age and impaired killing of <i>Salmonella</i> opsonised	170

	with acute and convalescent sera	
7.3	Blood cell killing of <i>S. Typhimurium</i> D23580 opsonised with HIV-infected acute and convalescent sera from African children with NTS bacteraemia	172
7.4	Blood cell killing of <i>S. Typhimurium</i> D23580 and anti- <i>Salmonella</i> IgG, IgA and IgM levels in sera from Malawian children	174
7.5	Anti- <i>Salmonella</i> antibody levels are elevated in convalescent sera compared to acute sera in children with NTS bacteraemia	178
7.6	Blood cell killing of <i>Salmonella</i> opsonised with diluted children's sera	180
7.7	Anti- <i>Salmonella</i> antibody titre and maximum dilutions of sera able to effect <i>Salmonella</i> killing	183
8.1	Sensitivity of Malawian invasive NTS isolates to bactericidal antibody	192
8.2	Antibody-dependent killing of NTS isolates by washed peripheral blood cells	194
8.3	Antibody-dependent oxidative burst and phagocytosis by peripheral blood cells following stimulation with invasive Malawian NTS isolates	195
8.4	Antibody and complement deposition on eleven Malawian NTS isolates	198

LIST OF TABLES

2.1	<i>Salmonella</i> isolates used throughout this study	35
5.1	Clinical data for study participants	98

ABBREVIATIONS

ADH	Adipic dihydrazide
BRS	Baby rabbit serum
BSA	Bovine serum albumin
Cfu	Colony forming units
CGD	Chronic granulomatous disease
ELISA	Enzyme linked immunosorbent ass
FACS	Fluorescence activated cell sorter
FcR	Fragment, crystallisable region
FITC	Fluorescein isothiocynate
IFN- γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KDO	3-deoxy-D-manno-octulosonic acid
LB	Luria Bertani
LPS	Lipopolysaccharide
NHS	N-hydroxysuccinamide
NTS	Nontyphoidal <i>Salmonella</i>
OAg	O-antigen
OMP	Outer membrane protein
PBS	Phosphate buffered saline
QECH	Queen Elizabeth Central Hospital
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen intermediates
Rpm	Revolutions per min
SPI-1	<i>Salmonella</i> pathogenicity island-1
TTSS	Type three secretion system
U	Units

PUBLICATIONS

Work discussed in this thesis has led to the publication of two manuscripts:

Appendix A.

O'Shaughnessy CM, Cunningham AF, MacLennan CA. The stability of complement-mediated bactericidal activity in human serum against nontyphoidal *Salmonella*.

PLoS One. 2012;7(11):e49147

Appendix B.

O'Shaughnessy CM, Micoli FM, Gavini M, Goodall M, Saul A, MacLennan CA. Affinity purification of antibody to the polysaccharide of *Salmonella* Typhimurium from human serum.

J Immunol Methods. 2013 Jan 31;387(1-2):199-210

CHAPTER 1. INTRODUCTION

1.1 THEME AND OVERVIEW

The theme of this thesis is the role of antibody in cell-mediated immunity to Nontyphoidal *Salmonella* (NTS). The requirement for opsonic antibody for immunity to NTS in HIV-infected African adults and in African children with NTS bacteraemia is investigated. The requirement of antibody and complement for blood cell killing of *Salmonella* compared to cell-free complement-dependent killing is studied as well as the susceptibility of invasive African isolates to antibody-mediated killing. This chapter will discuss *Salmonella* and the epidemiology of NTS infections. The pathogenesis, host immune response to infection and the development of vaccines for protection against *Salmonella* will also be discussed.

1.2.1 *SALMONELLA*

Salmonella is a genus of Gram-negative, rod shaped bacteria. They are approximately 0.7 to 1.5 μm in diameter and 2.0 to 5.0 μm in length. They are classed as chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. *Salmonellae* are mostly motile as they possess flagella which project in all directions.

1.2.2 TAXONOMY

The genus *Salmonella* is classified as a member of the Enterobacteriaceae family (1). The genus is divided into two species; *Salmonella bongori* and *Salmonella enterica*. *S. bongori* is largely restricted to cold-blooded animals (2). *S. enterica* is divided into six subspecies, based on genome phylogeny and biochemical and antigenic characteristics: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), hotenae (IV), and indica (VI). Within these six subspecies are more than 2400 serotypes (3). These serotypes are classified using the Kauffman-White scheme, according to O (carbohydrates associated with LPS) and H (flagellar proteins) antigens (4).

1.2.3 SALMONELLAE AS PATHOGENS

The majority of serovars which cause human disease, including Typhimurium, Enteritidis, Paratyphi and Typhi are within *S. enterica* enterica (subspecies I) (5). Infection with each of these serotypes results in a different disease state, which is dependent on the infecting serotype, the host species and the host's immune state. Infection with typhoidal *Salmonella* (*S. Typhi*, *S. Paratyphi* A, B and C) cause enteric fever, whereas infection with nontyphoidal *Salmonellae* (including *S. Typhimurium* and *S. Enteritidis*) cause gut-associated gastroenteritis and sometimes invasive bacteraemia. Some serotypes are restricted in terms of the hosts they infect. For example, *Salmonella Typhi* and *S. Paratyphi* A are host-restricted to humans and cause typhoid fever (1) and *S. Gallinarum* is host restricted to chickens, and causes typhoid-like disease (6). Some serotypes such as *S. Typhimurium* are promiscuous as they are able to infect different

animals as well as humans. In mice, *S. Typhimurium* infection causes a fatal enteric fever with symptoms similar to those in humans infected with *S. Typhi* (7). However, in humans, infection with *S. Typhimurium* usually results in enterocolitis, and in rare cases develops into a systemic infection.

1.3 EPIDEMIOLOGY OF NTS INFECTIONS

1.3.1 *SALMONELLA* INFECTIONS IN DEVELOPED COUNTRIES

The global burden of NTS gastroenteritis is estimated to be 93.8 million cases, resulting in 155,000 deaths annually (8). NTS has been identified as the leading cause of death from foodborne illness in the United States of America (9). Salmonellosis is usually a foodborne zoonosis with foods such as poultry, eggs, milk, fruit and vegetables implicated in transmission (10;11). Symptoms of NTS enterocolitis appear 12 to 72 hs after ingestion (12), and infected individuals experience mild, self-limiting gastrointestinal illness involving diarrhoea, abdominal cramps, fever, nausea and vomiting (13;14). In industrialised countries, development of blood stream infections are rare (15) except in immunocompromised individuals (16).

1.3.2 *SALMONELLA* INFECTIONS IN AFRICA

It is estimated that there are 50 cases of *Salmonella Typhi* per 100,000 people per year in Africa (17) and *S. Typhi* is the predominant invasive serotype in North Africa. In sub-Saharan Africa, NTS bacteraemia is a major public health problem (18-21). The burden of NTS infection is estimated to be 175-338 cases per 100,000 children aged 3-5 years

(20;22;23) and 2000-7500 cases per 100,000 HIV-infected adults (19;24;25). *Salmonella* serovars Typhimurium and Enteritidis are most frequently associated with diarrhoeal and bloodstream infections in Africa (26-28) (Figure 1.1). The mode of transmission is unknown, but it is thought that person-to-person transmission is important (13;29).

In African adults, invasive NTS disease is associated with HIV-infection (30). In a study in Malawi, 92-96% of febrile adults admitted to hospital with NTS bacteraemia were HIV-positive (31), and 80% had peripheral blood CD4 counts less than 200 cells/ μ l (30;31). Case fatality rates for HIV-infected adults with NTS bacteraemia are reported to be 47% (30). Recurrent infections are common in HIV-infected adults, with 22-44% of patients having further NTS infections, usually within 2-3 months (16;32). These recurrent infections are associated with recrudescence rather than re-infection (33), and a decline in recurrence is observed once anti-retroviral therapy is commenced (34).

Infection with NTS is also common in African children below 2 years of age (20;28), with less than one in five cases HIV-associated (26). The incidence of NTS bacteraemia is high with a mortality rate of 24% despite treatment with antibiotics (20;28;35). NTS can also cause meningitis (36), with the fatality rate increasing to 50% (31;37).

1.3.3 CLINICAL PRESENTATION

A lack of specific clinical presentation of invasive NTS infection makes diagnosis difficult (28). Patients often present with a febrile systemic illness (38;39), but gastrointestinal

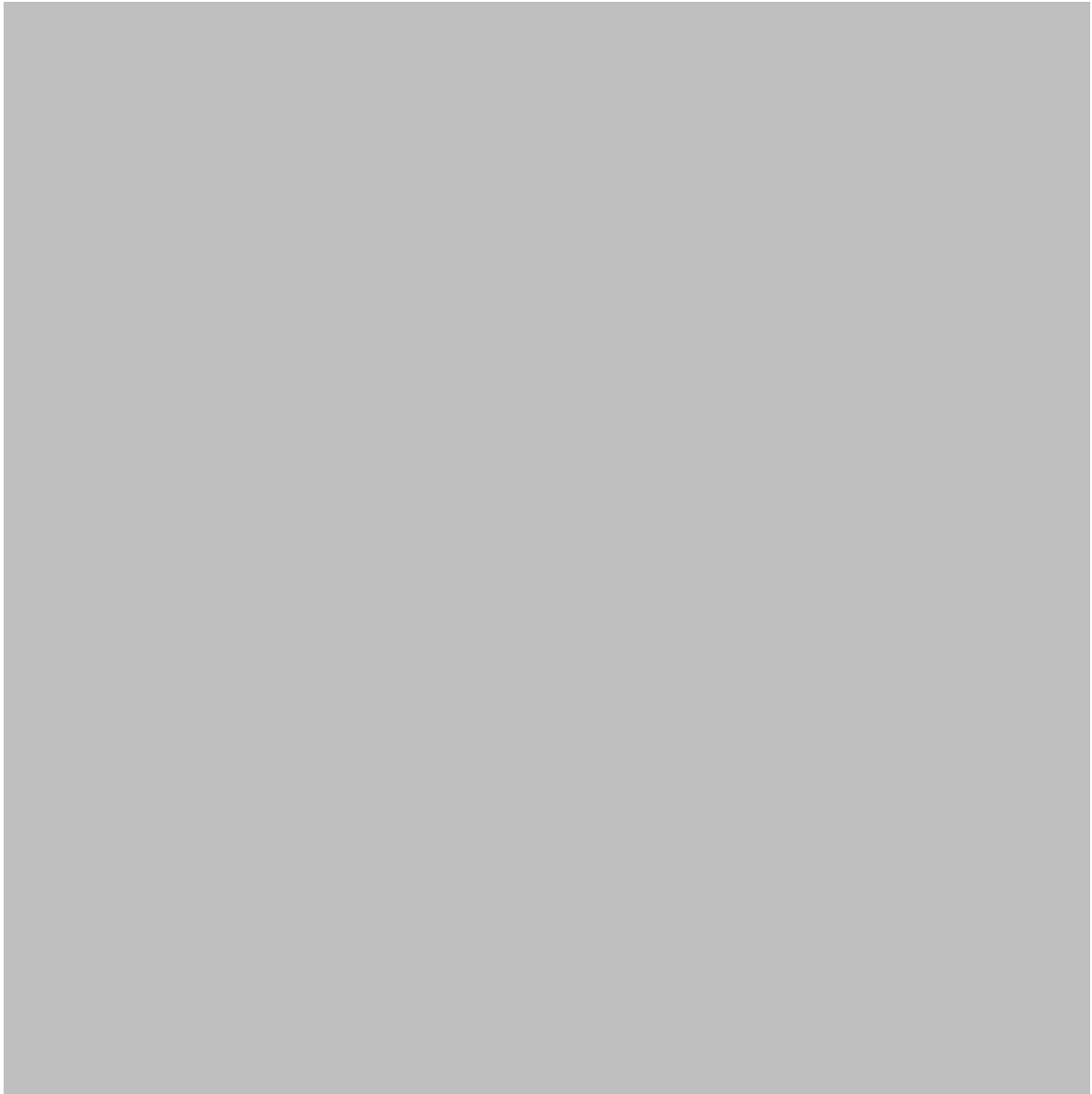


Figure 1.1 Map of Africa highlighting blood stream infection studies.

Results of a meta-analysis of studies investigating the cause of blood stream infections (BSI) in African children and adults (Figure taken from (24)).

symptoms are often absent (40) (Figure 1.2). Given the high incidence of malaria and pneumonia in sub-Saharan Africa, and that the clinical presentations for these diseases can overlap (39;41;42), mis-diagnosis is common. A blood culture is required for correct diagnosis of bacteraemia, but many hospitals and clinics lack such facilities.

1.3.4 TREATMENT

Antimicrobials are commonly prescribed to treat NTS bacteraemia, but multi-drug resistance is a growing problem. In Malawi, 90% of invasive NTS isolates are resistant to ampicillin, chloramphenicol and cotrimoxazole (31;43). Alternative antibiotics such as cephalosporins and fluoroquinolones are now prescribed, though these are expensive and drug-resistance against these antibiotics is likely to develop (44). Therefore, it is essential that a vaccine which provides protection against NTS is developed.

1.4 RISK FACTORS FOR INVASIVE NONTYPHOIDAL *SALMONELLA* DISEASE IN AFRICA

A high incidence of malaria, HIV and malnutrition are common in Africa and these together with young age are associated with the high disease burden of non-typhoidal salmonellosis in Africa. A significantly higher number of cases of NTS infection are observed during the wet season compared to the dry season, (43;46;47) suggesting that water sanitation is a risk factor for NTS disease. However, the prevalence of malaria and malnutrition also increase during the rainy season (18). Overcrowding, limited access



Figure 1.2 Clinical presentations of invasive NTS disease in African children and adults.

A lack of blood culture facilities and an absence of specific clinical presentation makes NTS difficult to diagnose (Figure taken from (45)).

to fresh water and poor sanitation are likely to further aid human to human transmission of NTS.

1.4.1 ENVIRONMENTAL RISK FACTORS

1.4.2 YOUNG AGE

Young age is a risk factor for mortality in Africa (28;48). A peak incidence of NTS bacteraemia is observed in African children aged between 6 and 24 months (20;49). Antibody passively acquired during the final trimester of pregnancy provides protection against NTS infection for the first few months of life, but as this maternal antibody titre wanes, infants are at increased risk of invasive disease as they are not yet able to produce their own antibody (50). A study of 352 cases of infants with bacteraemia at the Queen Elizabeth Central Hospital (QECH), Malawi in 2003-2004 found that NTS bacteraemia was commonest in children between 4 and 24 months of age which corresponded with an absence of antibody (50). An observed decrease in incidence in older children correlated with an increased acquisition of antibody (50). This supports the hypothesis that antibody is an important mechanism of protection against NTS.

1.4.3 MALARIA AND MALARIAL ANAEMIA

The association between malaria and invasive salmonellosis is well established. It was observed in British Guiana in the 1920's that NTS septicaemia was common during malaria outbreaks (51). A similar association has since been reported in children from

Nigeria, the Gambia and Malawi (16;35) where cases of malaria parasitaemia were significantly higher in children with bacteraemia caused by NTS compared to those with bacteraemia caused by other pathogens (28).

NTS bacteraemia is also associated with anaemia in young children in Africa (28;52;53). Severe malarial anaemia, where plasma haemoglobin levels drop below 5 g dl⁻¹, is particularly linked with NTS bacteraemia (54). One proposed mechanism for the association, is that malaria causes haemolysis of infected red blood cells which results in anaemia and in the release of free haemoglobin-derived heme from red blood cells (55). Free heme has cytotoxic effects and, to counteract this, host cells induce heme oxygenase-1 (HO-1) which removes heme and heme-derived metabolites (55). Phagocytosis and the subsequent oxidative burst are crucial mechanisms for destroying *Salmonella*. However, the activity of HO-1 suppresses the oxidative burst activity of granulocytes, consequently producing a niche for bacterial replication and overall increasing host susceptibility to NTS (56).

1.4.4 HIV

AIDS was first linked with *Salmonella* infection in the 1980s, at the outset of the AIDS pandemic (57;58). NTS has since been identified as the commonest blood stream bacterial isolate from febrile adults admitted to hospital in HIV-prevalent areas of Africa (35;38;59). Three immunological defects in HIV-infected adults have so far been identified which can help explain this association (45). Firstly, a loss of Th17 cells from

the gut mucosa has been identified in HIV-infected patients, leading to a failure to maintain the epithelial barrier and easier dissemination of bacteria from the gut into the blood stream (60). Secondly, an excess of antibody which targets the O-antigen chain of LPS on *Salmonella* Typhimurium has been observed in a proportion of HIV-infected African adults, and it is thought that the excess antibody blocks or diverts the access of other bactericidal antibody to the bacteria's surface (61). Finally, dysregulated cytokine production, particularly of TNF- α , IL-10 and IL-12 has been noted in HIV-infected adults (62), which would explain the intracellular persistence of the bacteria and the observed recurrent infections.

Although salmonellosis in African children was a common problem before the HIV epidemic, an association between HIV infection and NTS bacteraemia has also been observed in children, with 20% of children presenting with NTS bacteraemia also testing positive for HIV (21;26).

1.4.5 MALNUTRITION

Over 50% of blood isolates from children admitted to QECH, Malawi, with bacteraemia and severe malnutrition were identified as NTS (28;31), highlighting the association between malnutrition and NTS. A similar finding was observed in Uganda, where bacteraemia was reported in 17-22% of malnourished children (63;64), of which NTS was the commonest cause. It is thought that the intestinal lining and therefore the

normal immune response is disturbed in malnourished children, thereby permitting easier access of bacteria into the bloodstream.

1.5 *SALMONELLA* PATHOGENESIS

S. Typhimurium infection in mice provides an insight into the likely pathogenesis of *Salmonella*. The severity and outcome of the infection is dependent on several factors, including; the virulence of the infecting strain, the dose, route of infection, genetic makeup as well as the immunological status of the host (65).

Salmonellae usually infect their host through the oral ingestion of contaminated food or water. A proportion of the ingested inoculum will survive the low pH environment of the stomach to enter the small intestine (Figure 1.3). Once in the intestine, *Salmonellae* invade the epithelial cells and M cells in the Peyer's patches using a type III secretion system (TTSS) encoded by genes within *Salmonella* Pathogenicity Island-1 (SPI-1) (66). The TTSS encodes a needle like complex which injects effector proteins (1) such as SopE1, SopE2 and SopB from the bacterial cell into the host cell cytoplasm (67). These proteins activate intracellular signalling cascades, cytoskeletal rearrangements and stimulate the uptake of the bacteria by macropinocytosis (68;69). *Salmonellae* which are deficient in these virulence effector proteins are still able to invade the mucosal epithelia of the host (70). These bacteria are taken up by activated dendritic cells which have been recruited from the blood to the site of infection. These dendritic cells sample



Figure 1.3 Distribution of *Salmonella* in the mouse model.

Salmonellae first invade epithelial and M cells and are engulfed by resident macrophages and dendritic cells. The bacteria initiate caspase-1 mediated cell death of the host macrophage, leading to production of interleukin – 1β and interleukin-18, mediating the recruitment of polymorphonuclear phagocytes (PMNs). The bacteria enter the mesenteric lymph nodes and blood and are captured by phagocytes in the spleen and liver forming pathological lesions (Figure taken from (74)).

the luminal contents and engulfed bacteria are then transported into the blood by the dendritic cells (71).

Regardless of the route used by the bacteria to cross the epithelium, an early local inflammatory response is induced (72). Invasion of the epithelial cells leads to the production of interleukin-1 β and IL-18 which mediate the infiltration of polymorphonuclear leukocytes into the intestinal lumen from the blood (72). As the neutrophils migrate through the epithelial layer, there is an accumulation of protein rich fluid in the intestinal lumen, causing diarrhoea, 8-72 hs after bacterial colonisation (73). Once across the epithelium, the bacteria are engulfed by macrophages (74) and spread to the mesenteric lymph nodes and then into the blood (75). A transient state of bacteraemia is formed, but *Salmonella* which have avoided killing by the phagocytes are rapidly opsonised by antibody and complement, and are efficiently phagocytosed by neutrophils and monocytes in the spleen and liver (76;77). The phagocytes induce killing of the ingested bacteria through the generation of reactive oxygen species (ROS) and reactive nitrostatic intermediates (RNI), activating lysosomal enzymes and defensins. This mechanism controls the spread of the bacteria and initially reduces the number of *Salmonellae*. However, *Salmonellae* are capable of intracellular survival. Several *Salmonella* virulence genes are upregulated upon entry into the phagosome (2;78) which modify the host environment, impair the activity of anti-microbial NADPH oxidase and allow the formation of a *Salmonella* containing vacuole where *Salmonella* can replicate, prior to initiating caspase-1 mediated apoptosis of the macrophage (79) and release into the extracellular compartment.

Within 24 hours, the number of bacteria within the spleen and liver increase. The bacteria grow within distinct foci, but once the number of bacteria reaches a certain threshold, the bacteria escape and spread through the organ and enter previously non-infected cells to form new infection foci (74). It is unclear how bacteria spread from one focus of infection to another (74). In this way, the number of killed bacteria is insignificant compared to the exponential increase in the number of bacteria distributed within the organ (80;81).

Within 72 hours, there is systemic bacterial spread and bacteria are present in large numbers within the blood. Endotoxic shock and subsequent death of the mouse follow (82). It is unknown if systemic *S. Typhimurium* infection in African children and HIV-infected adults follows the same process.

1.6 *SALMONELLA* VIRULENCE AND RESISTANCE TO KILLING

During pathogenesis, *Salmonellae* use numerous mechanisms to both evade the host immune system and manipulate immune cell functions (83). Successful colonisation of the host depends on the expression of virulence proteins by *Salmonella* and also on the ability of *Salmonellae* to survive within the macrophage (84). Mutant strains of *Salmonella* which are not able to grow within macrophages are avirulent (84). *Salmonellae* have approximately 4500 genes and over 100 of these genes have been implicated in virulence (85;86). Variations in genes encoding for surface structures such as LPS and flagella, as well as for factors which modify host cell physiology affect

the virulence of the bacteria (5), and offer an explanation as to why some serotypes have increased virulence.

1.6.1 *SALMONELLA* PATHOGENICITY ISLANDS

Salmonella pathogenicity islands (SPIs) are DNA segments encoding important virulence genes. They are the product of multiple horizontal gene transfers from other bacteria during the course of evolution (87). *Salmonellae* have numerous SPIs, though SPI-1 and SPI-2 are the most important as they are central to *Salmonella* pathogenesis and virulence. SPI-1 is required for the invasion of non-phagocytic host cells, namely intestinal epithelial cells. SPI-1 encodes for a TTSS which transports effector proteins into the cytosol of the host cell, resulting in rearrangement of the cytoskeleton and the uptake of the bacteria into a membrane bound vesicle (88).

Unlike SPI-1 which is present in the genome of all species of *Salmonella* (89), SPI-2 is only present in *S. enterica*. SPI-2 also encodes TTSS apparatus and effector proteins which modify the intracellular environment within the host macrophage and consequently enhance *Salmonella* survival (90). The SPI-2 effectors direct the formation and maturation of the *Salmonella* containing vacuole (SCV), and also impairs the recruitment of NADPH oxidase to the phagosome (90).

1.6.2 LPS

The lipopolysaccharide (LPS) molecule of *Salmonella* plays an important role in infection (91). LPS is known to protect bacteria from serum complement killing. Rough

mutants, which have either no O-antigen or a truncated O-antigen, are more sensitive to bactericidal antibody and complement than the corresponding smooth strain (92). The length of the LPS O-antigen can also influence the interaction with complement and macrophages (93) and the size and distribution of the O-antigen can determine serum resistance (94). It is possible that LPS confers resistance to complement-dependent killing through diverting the deposition of membrane attack complex away from the bacterial surface (95).

1.6.3 PHOP/Q

PhoP/Q is an important two-component regulator of *Salmonella* virulence (96). It controls the expression of over 40 virulence genes. PhoP controls adaptation to the intraphagosomal environment and PhoQ is a sensor protein that is responsive to antimicrobial peptides, acid pH and low Mg^{2+} concentrations (97). Together they control the expression of genes and thus proteins involved in the formation of SCVs, macrophage-induced apoptosis, LPS regulation (98) and resistance to acidic pH (99) and antimicrobial peptides (100).

1.7 IMMUNITY TO *SALMONELLA*

Salmonellae are facultative intracellular organisms which are adapted to survive within macrophages (84) but are also capable of extracellular growth in the blood. Immunity against NTS is complex (101;102) and different stages of infection require collaboration

between different mechanisms of innate and acquired immunity, and involve both cell-mediated and humoral immunity (Figure 1.4).

The course of a primary sub lethal infection in the mouse model can be divided into four phases: phase 1 which involves the clearance of bacteria from the blood, phase 2 which corresponds to exponential growth of the bacteria in macrophages, phase 3, suppression of bacterial growth associated with the onset of the adaptive immune response and phase 4, the clearance of remaining organisms from the tissues (102).

1.7.1 PHAGOCYTES

Phagocytes express many different pattern recognition receptors that permit the recognition of pathogen microbial components. Toll-like receptor-4 (TLR-4) is a pattern recognition receptor which in conjunction with CD14, an LPS binding protein (103), recognises LPS on *Salmonella*. Flagellin is detected by TLR-5. TLR-4 knockout mice are more susceptible to infection, highlighting the importance of this interaction to the host (104;105).

During the first phase of infection, the majority of ingested *Salmonellae* pass through the epithelial cells in the gut and enter the blood stream, where phagocytes play a central role in controlling the infection. Neutrophils are the first type of phagocyte to

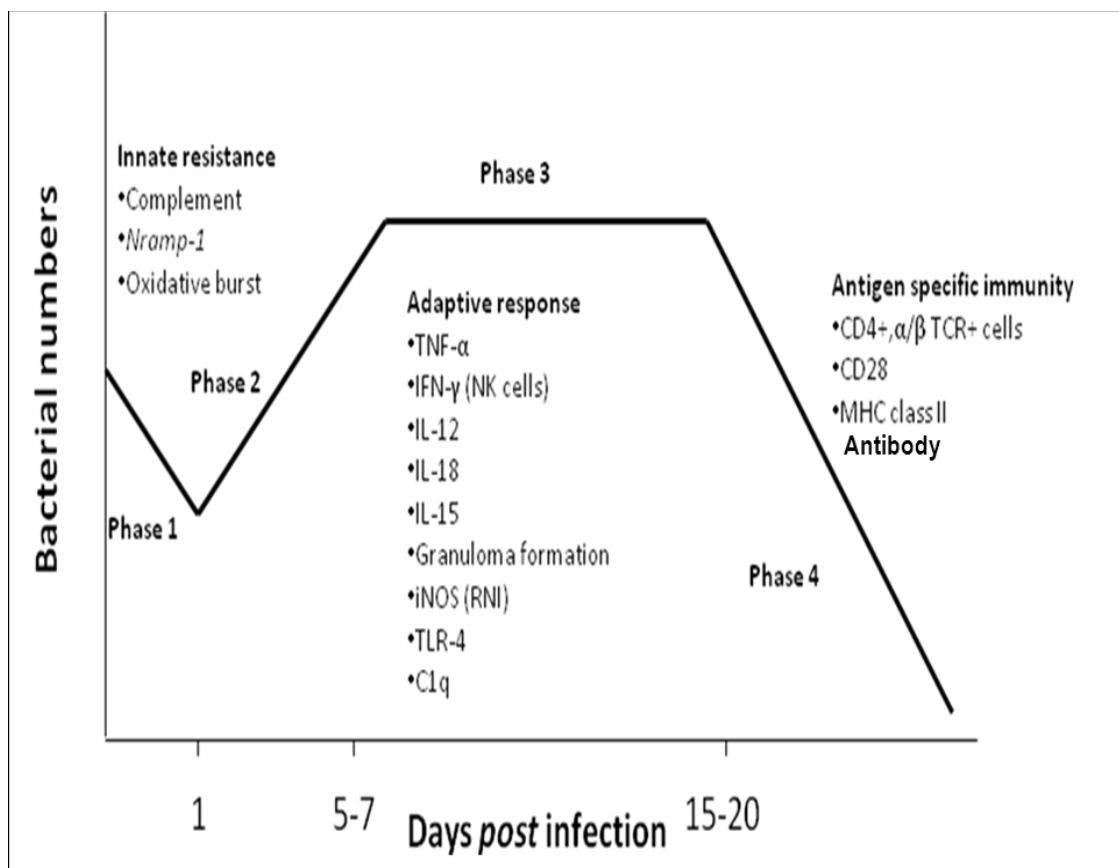


Figure 1.4 Phases of primary *Salmonella* infection.

The solid line shows the course of sub lethal infection in wild-type immune-competent mice and the immune mechanisms involved during each phase of infection. (Figure taken from (102)).

recognise and engulf opsonised *Salmonella*, hence their importance in the initial stage of infection. Most of the bacteria are cleared from the blood within 1-2 hours (76) through opsonisation with antibody and complement and subsequent uptake by phagocytes. The bacteria are phagocytosed through a process in which the bound pathogen is surrounded by the cell membrane and engulfed in a membrane-bound vesicle called the phagosome

Once within the cell, macrophages are able to kill, or at least limit the replication of intracellular bacteria through the production of antimicrobial peptides, lysosomes and toxic reactive oxygen and nitrogen species via the respiratory burst phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS) (90;106). The respiratory burst oxidase, NADPH oxidase, is a membrane bound enzyme complex comprised of gp91 and p22 subunits, which form cytochrome b₅₅₈, and cytosolic components; p40, p47, p67 and Rac1 proteins which assemble in the cytoplasmic membrane upon phagocyte activation (106). This enzymatic system is responsible for reducing molecular oxygen to superoxide, a cytotoxic precursor of oxygen radicals (107;108), in a process of rapid oxygen consumption, known as the respiratory burst. The products of the respiratory burst are toxic to the bacteria. The initial respiratory burst is followed by a longer, more sustained bacteriostatic production of nitric oxide (109;110) by inducible nitric oxide synthase (iNOS), which catalyses the production of NO from oxygen, NADPH and L-arginine. Together these mechanisms maximise the initial reduction in bacterial numbers but minimise the side effects to the host (106).

The importance of the respiratory burst in killing ingested bacteria is demonstrated in patients with chronic granulomatous disease (CGD)(106). CGD is an inherited disorder in which mutations occur in X-linked (gp91) or autosomal (p22, 47, 67) genes encoding subunits of the NADPH oxidase. Patients with CGD have recurrent infections and develop septicaemia as although they can phagocytose the invading bacteria, they are unable to mount a successful respiratory burst which would kill the bacteria, or acidify the lysosome so that lysosomal enzymes can work at their optimal pH. Recurrent infections occur from birth (111) and IFN γ is administered to reduce the number of severe infection, with a bone marrow transplant often required. *Salmonella* has been identified as the second most prevalent cause of frequent and severe infections in CGD patients (112).

1.7.2 CYTOKINES

Cytokines such as TNF- α , and Interleukin-1 (IL-1), IL-6, IL-12, IL-18 are produced by macrophages, and other secreted cytokines such as IL-10 and IL-4, and TNF- α and IFN- γ are produced by Th2 and Th1 cells respectively. The production and release of these cytokines is a hallmark of invasive *Salmonella* infection (113) and are important for defence against disease.

1.7.2.1 TNF- ALPHA

TNF- α is one of the first cytokines to be released by macrophages during *Salmonella* infection. Both TNF- α and IFN- γ have been shown to play an important role in

preventing systemic infection by wild type *S. Typhimurium* in orally inoculated mice (114) and pre-treatment with exogenous TNF- α of susceptible mice infected with *Salmonella* resulted in increased survival (115). Furthermore, TNF- α receptor p55 deficient mice succumb earlier to virulent *Salmonella* challenge (116), highlighting the importance of this cytokine in host defence.

1.7.2.2 IFN- γ

The internalisation of *Salmonella* by macrophages induces the production of IL-12 which stimulates NK cells and T cells to produce IFN- γ (117) which activates the macrophages and enhances anti-bacterial activity (118). Gordon *et al* showed that priming with IFN- γ increased the numbers of internalized *Salmonella* by human monocyte-derived macrophages compared to un-primed cells (119). The importance of the role of IFN- γ in immunity to NTS is highlighted by the observed increased susceptibility to recurrent NTS infections in patients who have CGD or genetic deficiencies in either the IFN- γ or in the IL-12/IL-23/IFN- γ axis (120;121). The administration of IFN- γ to infected mice, a few days after challenge with a virulent strain reduced the severity of the infection (122) and cessation of this anti-IFN- γ treatment (123) or depletion of IFN- γ using anti-IFN- γ antibody enhanced the severity of the infection (124).

1.7.2.3 IL-12

IL-12 strongly induces IFN- γ production by lymphocytes and is therefore a key cytokine in immunity against *Salmonella*. The importance of IL-12 has been demonstrated in mice and humans. Neutralisation of IL-12 increases the number of *Salmonellae* found in the spleen of mice and results in impaired survival (125), while treatment with exogenous IFN- γ increases host survival (126). Patients with IL-12 or IL-12 receptor deficiencies are highly susceptible to severe *Salmonella* infections (127;128) and subcutaneous IFN- γ is prescribed along with antibiotics to treat infections (127;128).

1.7.2.4 IL-15

IL-15, which has very similar biological activities to IL-12 (129;130), is produced by activated macrophages and monocytes and is involved in the activation of NK cells and $\gamma\delta$ -T cells in mice (131;132).

1.7.2.5 IL-18

IL-18 is also produced by monocytes and macrophages (133) and together with IL-12, induces both IFN- γ and TNF- α production by human NK and T cells (134;135). In mice, neutralisation of IL-18 results in increased bacterial growth in the spleen and liver and diminished host survival, and treatment with IL-18 decreases the number of bacteria found in these tissues and increases host survival (135).

1.7.2.6 IL-10 AND IL-4

IL-10 has an inhibitory effect on host immunity to NTS by antagonizing the effect of IFN- γ on macrophages. Impaired killing of *Salmonella* by macrophages is observed in mice expressing the IL-4 gene (136) and increased resistance to *Salmonella* infection is observed in IL-4-deficient mice compared with wild-type mice (137).

1.7.3 T CELLS

Although the innate immune response controls the initial infection, adaptive immunity and a *Salmonella*-specific T-cell response is required to eradicate the remaining bacteria and provide protection against subsequent infections (1). *Salmonella* infection induces both CD4⁺ T cells and CD8⁺ T cells, with CD4⁺ T cells, which mediate protection by producing cytokines such as IFN- γ , playing a central role in clearance of the bacteria from tissues, with little contribution from CD8⁺ T cells. The depletion of CD4⁺ T cells, rather than CD8⁺ T cells has a greater effect on both control of the primary infection and protection induced by vaccination with attenuated strains of *Salmonella* Typhimurium (82). Furthermore, protection against infection was increased when CD4⁺ T cells from vaccinated mice were transferred into naïve mice compared with when CD8⁺ T cells were transferred (138-140) reinforcing the importance of CD4⁺ T cells over CD8⁺ T cells. Furthermore, in the absence of CD4⁺ T cells, phagocytes are unable to clear bacteria from their cells due to impaired antibacterial function, causing severe infection (140).

The *Salmonella*-specific T cell response is directed against *Salmonella* protein antigens such as porins and flagellin (102). McSorley *et al* have shown that the flagellar filament protein FliC, is a major target of CD4⁺ T cells during both primary and secondary infection and can protect naïve mice from fatal *Salmonella* infection (141). CD8⁺ T cells control *Salmonella* infection through lysing infected macrophages with poor bactericidal activity (140) thus, causing the release of bacteria into the blood, and exposing them to antibody and complement. Cytotoxic CD8⁺ T cells also contain granulysin which has antimicrobial activity against *Salmonella* (142).

$\gamma\delta$ -T cells may also play a role in immunity to *Salmonella* infection, although, mice deficient in $\gamma\delta$ -T cells are able to control systemic infection with an attenuated strain of *S. Typhimurium* (122). Mice with defects in $\alpha\beta$ -T cells infected with attenuated strains of *Salmonella* Typhimurium fail to control infection though and develop chronic disease, suggesting that $\alpha\beta$ -T cells are more important than $\gamma\delta$ -T cells (1).

1.7.4 B CELLS

B cells display a range of functions in the immune response against infection with *Salmonella*, including antigen presentation and the production of antibodies and cytokines. B cells contribute to early protection in mice challenged with *Salmonella*, and together with antibodies, provide protection against secondary challenge. B cell-deficient mice demonstrate impaired resistance to primary *Salmonella* infections, caused by a defect in antibody production (102). Furthermore, impaired production of

Th1 cytokines, IL-12 and IFN γ is also observed in B cell-deficient mice, resulting in the failure to control virulent *Salmonella* infection (143).

1.7.5 COMPLEMENT

The complement system is an important component of the innate immune system and plays an essential role in host defence against pathogens (77). It is composed of a series of heat-labile serum proteins, which can be activated via three different pathways (Figure 1.5) to generate a series of effector proteins which help to destroy the invading pathogen. The classical pathway is activated by the binding of C1q (144) to IgM, IgG1 and IgG3 bound to the bacterial surface. The alternative pathway is continuously activated due to a low level of spontaneous hydrolysis of C3. The lectin pathway is activated by mannose-binding lectin binding to mannose residues on the bacteria's surface. Invasive strains of *Salmonella* Typhimurium activate complement via the alternative and classical pathways but do not activate mannose-binding lectin bactericidal complement (50). Although the pathways are activated differently, the pathways converge to activate C3 convertase which cleaves C3. The fragment C3b is a major opsonin and can covalently bind to the bacteria enhancing phagocytosis (145) and destruction of the bacteria. Fragments C3a, C4a and C5a are inflammatory activators which recruit and activate phagocytes. C5b triggers the assembly of fragments C5b-C9 into the membrane attack complex (MAC) which is deposited and inserted into the bacterial cell membrane (146). Consequently, the lipid bilayer and cellular homeostasis is disrupted and the bacteria is destroyed (95). It is essential that

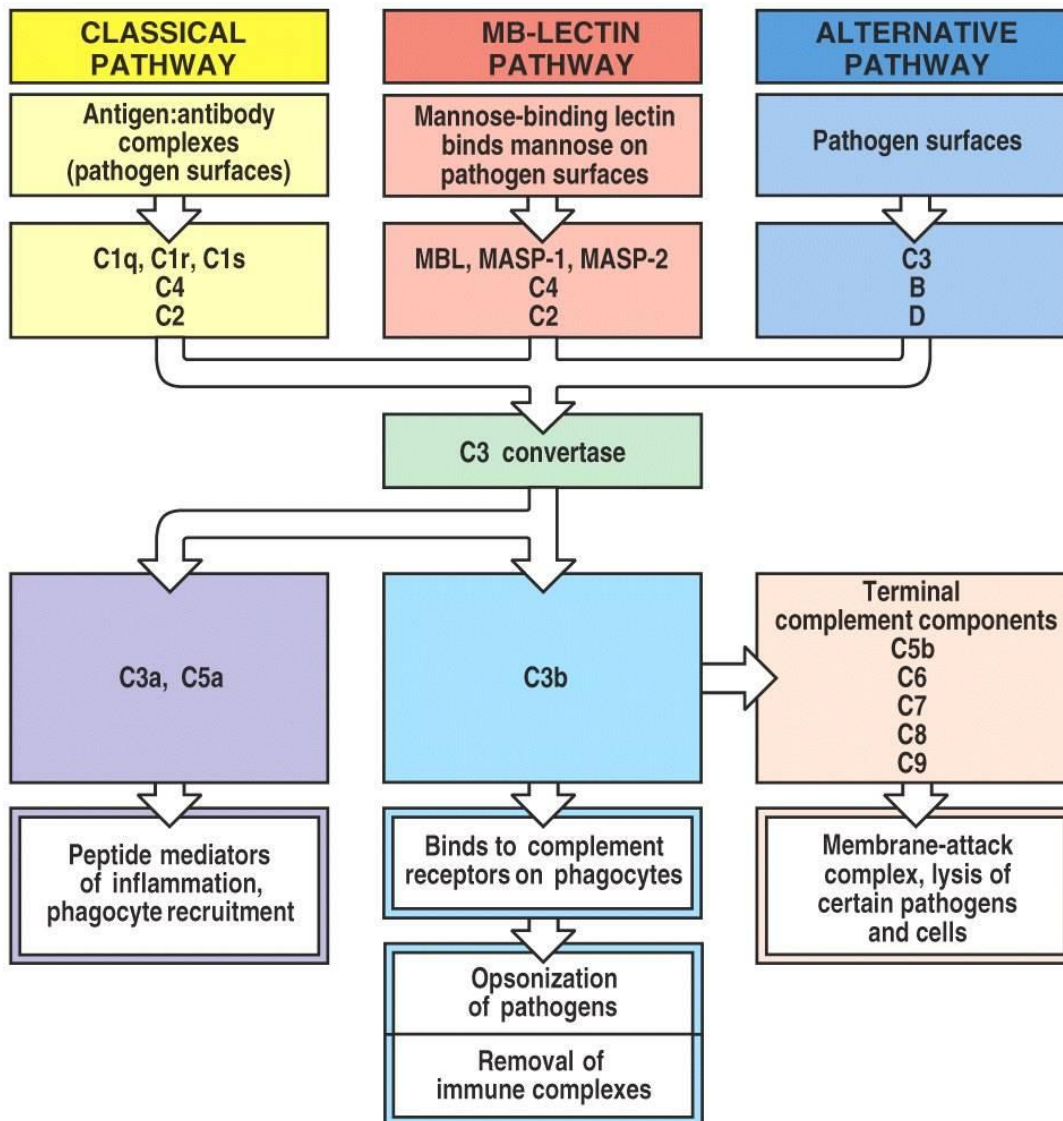


Figure 2-19 Immunobiology, 6/e. (© Garland Science 2005)

Figure 1.5 Activation and function of the three complement pathways.

All three pathways that activate the complement cascade converge at the formation of C3 convertase. C3 convertase cleaves C3 into components C3a and C3b, ultimately leading to opsonisation of the pathogen, release of inflammatory mediators, and the formation of the membrane attack complex. (Figure taken from (154)).

complement activation is carefully regulated. Factor H is the main inhibitor of the alternative pathway and both C1 inhibitor and C4bp inhibit the classical pathway (147).

1.7.6 ANTIBODY

Although NTS are intracellular bacteria they are also capable of extracellular growth in the blood, and antibody plays a key role in preventing extracellular growth and controlling infection (50) (Figure 1.6). Adoptive transfer studies in mice have shown that optimal protection against *Salmonella* infection is conferred by both antibody and T cells (148;149). Antibody to *Salmonella* has also been shown to reduce bacteraemia in mice, as well as prevent primary infection and impede hematogenous spread of *Salmonellae* (150). Young African children who lack antibody to NTS, but have functional complement are more susceptible to NTS infection (50). When sera from these children are supplemented with IgG, killing is restored, indicating the importance of bactericidal antibody in controlling extracellular growth in the blood (50). Furthermore, an absence of antibody in these young African children impedes oxidative burst and cellular killing of NTS and when exogenous IgG is added, optimal killing of *Salmonella* is observed (151). The importance of antibody in immunity to *Salmonella* is also highlighted by patients with primary immunodeficiencies (152). For example, persistent diarrhoea, caused by *Salmonella*, was reported in patients with X-linked agammaglobulinaemia (153).

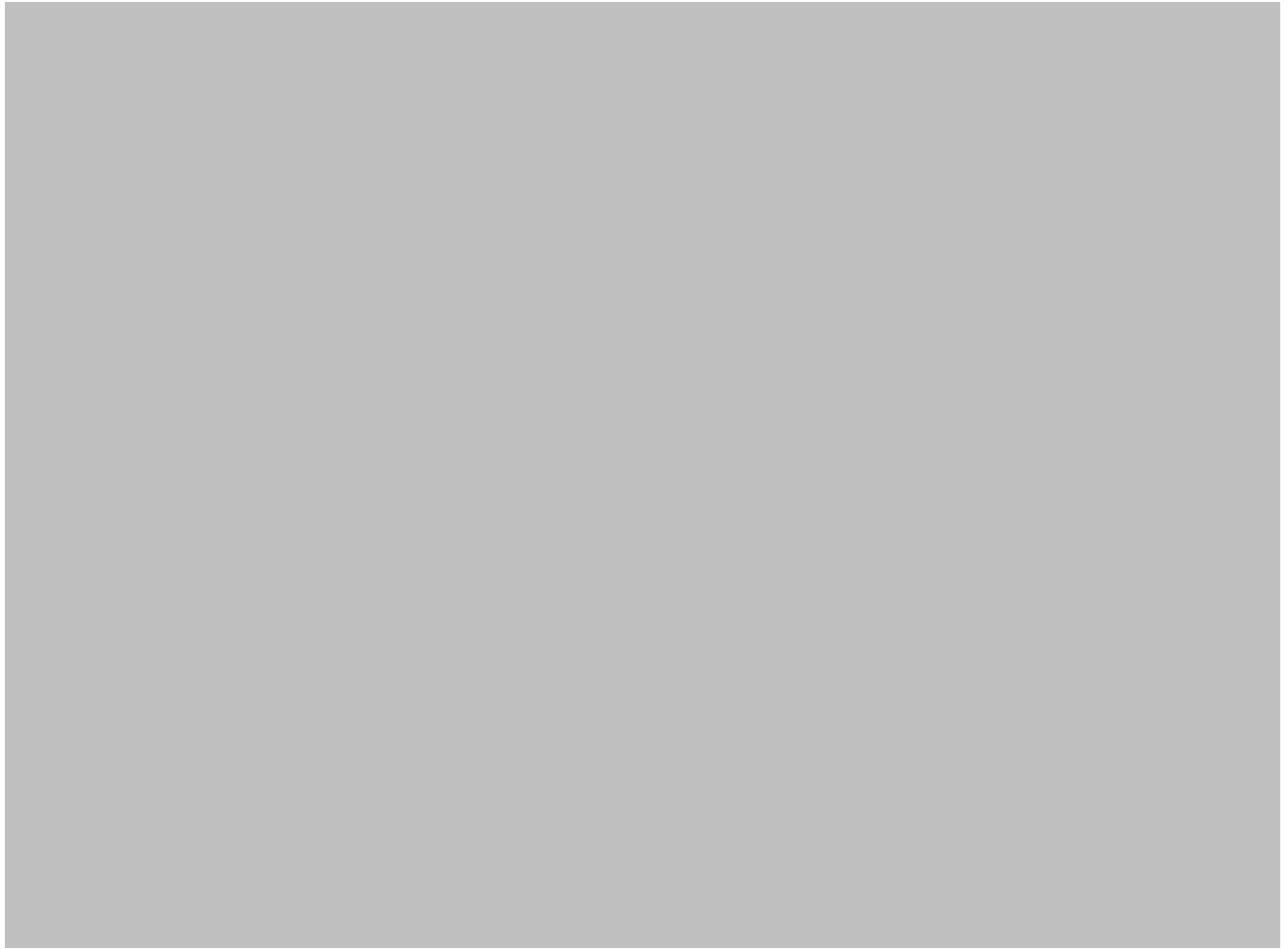


Figure 1.6 The key immunological factors that control the intracellular and extracellular phases of infection in humans.

Salmonellae grow within the phagocytic cells and cell-mediated immunity enhances the antimicrobial functions of the host phagocyte, such as the production of reactive oxygen intermediates, killing a proportion of the ingested bacteria but also releasing some bacteria into the extracellular space (80;81). Once in the extracellular space, antibody and complement can be deposited on the bacteria, opsonising it and facilitating uptake by phagocytes expressing the corresponding Fc receptor, enhancing bacterial internalisation and forming new infection foci. Furthermore, when in the extracellular space, antibody-dependent, complement-mediated killing of *Salmonella* can occur, and in humans significant killing of invasive NTS is observed via this mechanism (Figure taken from (74)).

Infection with NTS initially induces IgM antibody, followed by IgG and IgA (102). Antibody is produced against a variety of antigens, including lipopolysaccharide, flagellin and outer membrane proteins (82;155). Studies in mice and rabbits have shown that antibody against LPS (156-159) and porins (160) is protective against challenge with virulent *Salmonellae*. There is evidence for a role for both bactericidal and opsonic antibody in immunity to *Salmonella* (95;161). Antibodies against NTS can be opsonic and bind to the pathogen thus facilitating recognition and uptake by phagocytes. Binding of the Fc region of the antibody to the FcR expressed on the surface of the phagocyte is key in activating the cell's effector functions (162). The IgG subclasses differ in their affinity for Fc receptors, and consequently the efficiency of the uptake of opsonised bacteria depends on the IgG subclass and the type of FcR available (163). IgG3 is the most effective opsonin, followed by IgG1 and IgG4 which all have a high affinity for FcγRI, and IgG2 is weakly opsonic but has a higher dependency on FcγRIIa than FcγRI (163). Enhanced bacterial uptake was observed when bacteria were opsonised with serum from immunised mice (145), and in African children, optimal uptake of bacteria is observed when *Salmonella* are opsonised with both antibody and complement (151). Antibody is also able to protect the host against *Salmonella* infection through activating the classical complement pathway and inducing complement-mediated lysis (95;161). IgM is more efficient than IgG and IgA in triggering complement-mediated killing of bacteria.

The majority of studies on immunity to *Salmonella* have focused on cell-mediated immunity to NTS. However, studies in both animals and humans also show a clear

requirement for antibody in immunity to NTS (50;148;149;151;152). It is likely that cell-mediated immunity controls the infection when the bacteria are residing within the phagocytes and humoral immunity controls the spread of bacteria once they are released from the cells and are in transit between cells (74). This suggests that a successful vaccine needs to induce both antibody and cell-mediated responses to NTS.

1.8 VACCINES AGAINST *SALMONELLA*

Following the recognition of the disease burden caused by NTS in Africa and the emergence of resistance to antibiotic treatment, there is a growing need for a cheap and effective vaccine which will protect against *Salmonella* infection. There are currently no licenced vaccines for *S. Paratyphi A*, *S. Typhimurium* or *S. Enteritidis* for use in humans. Live attenuated *Salmonella* vaccines have been shown to induce increased protection than killed vaccines (164). However, live vaccines can be potentially dangerous for patients with immunodeficiencies who are more susceptible to *Salmonella* infection. This could be an issue in Africa, where immune suppression and dysregulation secondary to HIV infection are common. In addition to this, the ideal live attenuated *Salmonella* vaccine strain should harbour multiple independent attenuating mutations to make them safer. Attenuated *S. Typhimurium* and *S. Enteritidis* strains with two attenuations in genes encoding for guanine biosynthesis and a protease regulator were tested in mice (165). The strains were found to be highly attenuated, but still immunogenic, and induced high titers of anti-LPS and anti-flagellin antibodies and protected against challenge with wild-type invasive strains (165). This suggests the

potential for an oral live-attenuated bivalent vaccine which would protect against *S. Typhimurium* and *S. Enteritidis*.

Over the last century, several vaccines which provide protection against typhoid fever caused by *Salmonella Typhi* have been produced. These vaccines have ranged greatly in efficacy and reactogenicity (166;167) but only two vaccines remain in use. These are the Vi vaccine; a subunit vaccine based on an unconjugated purified Vi polysaccharide of *S. Typhi* (168) which is licensed for children over 2 years of age (169). In adults, a single intramuscular dose of Vi vaccine provides 70% protection, falling to 50% after 3 years (170). The other vaccine in current use is the Ty21a vaccine, an oral live attenuated *galE* mutant (171). This is not licensed for use in children under 6 years old (172-174). Furthermore, it requires 3 or 4 doses and its efficacy is only 51% (167).

To improve the antibody response and immunological memory, vaccine antigens are often conjugated to a carrier protein, such as diphtheria toxoid, tetanus toxoid or CRM₁₉₇ (a non-toxic mutant of diphtheria toxin). Conjugation of the Vi polysaccharide to non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA) enhanced the immune response and was shown to be protective even in 2-5 year old children (175;176). However, it is not yet licenced for use in humans. Recently, the O-polysaccharide of *S. Typhi* has been conjugated to diphtheria toxoid, and it induced significantly higher levels of IgG in immunised mice than the polysaccharide alone (177).

Novartis Vaccines Institute for Global Health have developed a Vi-CRM₁₉₇ conjugate vaccine, using Vi from *Citrobacter* which is immunologically indistinguishable from *S. Typhi* Vi but is from a safer source as it is a BSL-1 category organism, compared to *S. Typhi* which is a BSL-3 category organism (178). This conjugate vaccine has been shown to be immunogenic in mice (179;180) and in European adults, inducing higher anti-Vi antibodies than the licenced Vi vaccine (181). However, only *S. Typhi* and *S. Paratyphi C* express Vi, so this vaccine will not protect against NTS.

It is still unclear exactly which antigens are responsible for protection against invasive NTS (75) particularly in Africa, and because a large number of different *Salmonella* serotypes are implicated in causing disease in humans, identification of common antigens is difficult. OmpD, a porin found in most *Salmonella* serovars but not *S. Typhi* is a possible vaccine candidate (182) and SseB, a secreted effector protein, has been identified as a prominent antigenic target in both murine salmonellosis and in bacteraemic African children (183). As well as identifying a suitable target antigen, vaccine developers need to consider that a successful vaccine against NTS for use in Africa, must induce protective immunity in infants as well as immunocompromised adults, be low cost, heat stable, easy to administer and ideally require a single dose.

1.9 AIMS AND OBJECTIVES

The aims of this thesis are:

1. To develop methods to better understand the role of opsonic antibody in immunity to invasive African isolates of NTS.
2. To examine how opsonic antibody, in particular, anti-LPS antibody, enhances or inhibits cell-mediated killing of NTS in HIV-infected Malawian adults.
3. To study the role of antibody in killing of NTS by phagocytic cells in Malawian children with acute NTS bacteraemia and in convalescence.
4. To investigate the sensitivity of Malawian NTS isolates to opsonic antibody and identify how this relates to sensitivity to bactericidal antibody.

CHAPTER 2. MATERIALS AND METHODS

2.1 ETHICAL APPROVAL

Ethical approval for the collection of blood and *Salmonella* isolates from bacteraemic African children, and blood from HIV-infected and non-HIV infected African adults was granted by The College of Medicine Research and Ethics Committee, University of Malawi. Ethical approval for the use of blood and serum samples collected in the UK was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham.

2.2 LABORATORY METHODS

2.2.1 BACTERIA

Strains of *Salmonella* enterica serovar Typhimurium and Enteritidis (listed in Table 2.1) which were used throughout this study were isolated from blood cultures from children presenting to Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi with bacteraemia caused by infection with nontyphoidal *Salmonella* between 2003 and 2004. In addition to these wild type strains, an LPS mutant, *galE*⁻, expressing a truncated LPS molecule lacking O-antigen was kindly provided by R. Kingsley, Wellcome Trust Sanger Institute, UK.

2.2.1.2 PREPARATION OF *SALMONELLA* ENTERICA SEROVAR TYPHIMURIUM STATIONARY PHASE CULTURE

Serovar	Isolate	Source
Typhimurium	D23580	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D24545	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D24871	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D25352	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D23005	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D24953	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D26104	Blood culture from human, Malawi 2003 - 2004
Enteritidis	D24954	Blood culture from human, Malawi 2003 - 2004
Enteritidis	D24359	Blood culture from human, Malawi 2003 - 2004
Enteritidis	D24793	Blood culture from human, Malawi 2003 - 2004
Enteritidis	D23682	Blood culture from human, Malawi 2003 - 2004
Typhimurium	D23580 <i>galE</i> ⁻	<i>galE</i> ⁻ mutant, D23580 parent isolate

Table 2.1 *Salmonella* isolates used throughout this study.

A single colony of bacteria was selected from a culture of *Salmonella* on an LB agar (Sigma) plate using a sterile loop, and inoculated into 10 ml LB broth (Sigma). This universal tube was incubated (loose capped) overnight at 37°C in an incubator (New Brunswick Scientific) resulting in *Salmonellae* stationary phase.

2.2.1.3 PREPARATION OF *SALMONELLA* TYPHIMURIUM IN LOGARITHMIC PHASE

100 µl overnight stationary phase *Salmonella* culture was added to 10 ml pre-warmed (at 37°C) LB broth (Sigma) in a tight capped universal tube, and incubated for 2 or 3 h depending on the concentration of *Salmonella* required, at 37°C on a rocker plate (Stewart Scientific) at 20 rpm. The required quantity of inoculum was centrifuged at 3300 x g in microfuge tubes for 5 min. The bacteria were washed twice by the addition of 1 ml Phosphate Buffered Saline (PBS) (Sigma), centrifuging at 3300 x g, and discarding the supernatant. The bacteria were re-suspended in PBS to give the desired *Salmonella* concentration. The precise concentration was determined with Miles and Misra serial dilution and plating on LB agar.

2.2.2 PREPARATION OF HUMAN SERUM

Blood was taken from healthy adult volunteers and left to clot at room temperature. Once sufficiently clotted, the blood was centrifuged for 20 min at 4°C, 4000 x g in a Megafuge (Hereus, Germany). The supernatant was centrifuged again for 20 min, at 4°C, before aliquoting into microfuge tubes. The aliquots were immediately stored at -80°C.

2.2.3 SERUM BACTERICIDAL ASSAY (SBA)

10 µl of washed *Salmonella* Typhimurium inoculum at a concentration of 1×10^7 cfu/ml (prepared as described in section 2.2.1.3) was added to 90 µl of serum either in a microfuge tube or 96 well round bottom plates (Sarstedt, Germany) to give a final concentration of 1×10^6 cfu/ml. Samples were incubated at 37°C on a rocker plate (20rpm) for 180 min. At 45, 90 and 180 min, 10 µl of each sample was serially diluted with 90 µl of PBS in a 96 well plate following the Miles and Misra method. A 10 µl aliquot of each serial dilution was plated in triplicate on LB agar. The agar plates were incubated at 37°C overnight, and the number of viable colonies counted and concentration of *Salmonella* calculated. By subtracting the original concentration of *Salmonella* from the concentration of *Salmonella* at each time point, the net growth or killing of *Salmonella* by serum was determined. An increase in bacterial count from the starting inoculum concentration indicated net bacterial growth and a decrease indicated net killing of bacteria (Figure 2.1).

2.2.4 PREPARATION OF WASHED BLOOD CELLS

Blood was taken from healthy adult volunteers, into a tube containing heparin, at a final concentration of 4 U per ml of blood. The blood was washed twice to remove endogenous complement and antibody with RPMI-1640 (Sigma) and centrifuged in a bench-top centrifuge at $250 \times g$, 4°C for 10 min. The washed blood cells were re-suspended in RPMI to the original blood volume.

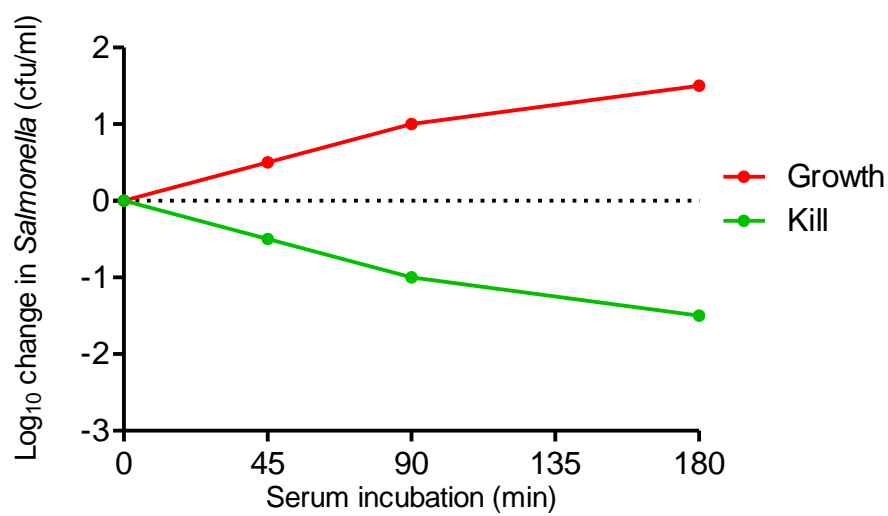


Figure 2.1 Serum bactericidal assay analysis.

Sample analysis of serum bactericidal assay showing bacterial growth (red line) over 180 min compared to the starting *Salmonella* concentration, and decrease in bacterial numbers, indicating killing of the bacteria (green line).

2.2.5 INTRACELLULAR BLOOD CELL KILLING ASSAY

To opsonize the bacteria, 10 µl of washed *Salmonella* Typhimurium inoculum at a concentration of 1×10^7 cfu/ml (prepared as described in section 2.2.1.3) was added to 90 µl of serum or PBS (as a negative control) in a microfuge tube, for 20 min at room temperature. 90 µl of washed blood was aliquoted into each microfuge tube, and 10 µl of opsonised *Salmonella* was added to give a final concentration of bacteria of 1×10^5 cfu/ml. Samples were gently vortexed and incubated at 37°C on a rocker plate, at 20 rpm, for 180 min. At 45, 90 and 180 min, 10 µl of each sample was serially diluted with 90 µl of PBS in a 96 well plate following the Miles and Misra method. A 10 µl aliquot of each serial dilution was plated in triplicate on LB agar. The agar plates were incubated at 37°C overnight, and the number of viable colonies counted and concentration of *Salmonella* calculated. By subtracting the original concentration of *Salmonella* from the concentration of *Salmonella* at each time point, the net growth or killing of *Salmonella* by blood cells was determined. An increase in bacterial count from the starting inoculum concentration indicated net bacterial growth and a decrease indicated net killing of bacteria.

2.2.6 QUANTIFICATION OF PHAGOCYTTIC ACTIVITY OF NEUTROPHILS AND MONOCYTES IN WASHED BLOOD IN RESPONSE TO STIMULATION WITH *SALMONELLA*

2.2.6.1 LABELLING OF *SALMONELLAE* WITH FITC

10 mg Fluorescein isothiocyanate (FITC) (Sigma) was dissolved in 10 ml PBS, giving a 1 mg/ml solution. This was incubated at room temperature in the dark for 10 min and

filter sterilized using a 0.2 µm filter. To FITC-label *Salmonella*, 2.5 µl of FITC solution was added to 100 µl *Salmonella* Typhimurium (grown for 3 h and re-suspended in PBS at a final concentration of 1×10^{10} cfu/ml and heat-killed by incubation at 72°C for 30 min) in a foil covered microfuge tube. The mixture was incubated on a rocker plate at 20 rpm, 37°C for 20 min. The FITC-labelled *Salmonellae* were washed twice with 1 ml PBS, centrifuged for 5 min at 3300 x g, and the supernatant aspirated each time to remove any excess FITC solution. The FITC-labelled *Salmonellae* were re-suspended in 100 µl PBS and stored in a foil-covered microfuge tube to protect from the light.

2.2.6.2 PHAGOCYTOSIS ASSAY

50 µl of washed blood (following the method outlined in section 2.2.4) was aliquoted into 5 ml BD FACS tubes and placed in an ice bath at 0°C for 10 min. 10 µl of FITC-labelled *Salmonellae* were opsonised with 90 µl of PBS (as a negative control) or serum, for 20 min at room temperature. 10 µl of opsonised-FITC-labelled-bacteria was added to the blood. For each sample there was a paired control tube which remained on ice, with the sample tube transferred to a water bath at 37°C. To stop phagocytosis, after the 10 min incubation, sample tubes were immediately placed into the ice bath and 50 µl of 0.16% trypan blue dye (Sigma) was added to quench the fluorescent signal from non-internalised bacteria. The tubes were mixed by vortexing. 1.5 ml of PBS was added to each tube and tubes were then vortexed and centrifuged at 250 x g, 4°C for 5 min. The supernatant was aspirated and the remaining cells were washed for a second time. 1 ml of 1 x FACS lysing solution (Becton Dickinson) (diluted 1:10 from 10 x stock with

deionised water) at room temperature was added to each tube to lyse the red cells, and the tubes were incubated at room temperature in the dark for 20 min. The tubes were then centrifuged at 250 x g, 4°C for 5 min and the supernatant discarded. The cells were washed for a second time with 1.5ml PBS and centrifuged, followed by aspiration of the supernatant as before. Finally, 100 µl of propidium iodide (Sigma) at a concentration of 20 µg/ml was added to each tube to identify non-viable cells during subsequent analysis, and the samples were incubated in the dark on ice for 10 min.

Data from samples was acquired using a FACS Calibur flow cytometer (Becton Dickinson) and Cellquest Pro software within 60 min. Non-viable cells were excluded by gating the negative population in the FL2 channel. Phagocytosing neutrophils and monocytes were gated according to forward and side scatter characteristics (Figure 2.2). The percentage of neutrophils and monocytes which have phagocytosed bacteria was determined for each sample from data acquired in the FL1 channel.

2.2.7 QUANTIFICATION OF OXIDATIVE BURST ACTIVITY OF NEUTROPHILS AND MONOCYTES IN WASHED BLOOD IN RESPONSE TO STIMULATION WITH *SALMONELLA* TYPHIMURIUM

This assay is adapted from the Bursttest kit (Orpegen Pharma, Germany). 10 µl of heat-killed *Salmonellae* (grown for 3 h and re-suspended in PBS at a concentration of 1×10^{10} cfu/ml, before heat-inactivating at 72°C for 30 min) was opsonized with 90 µl of serum or PBS for 20 min at room temperature. 50 µl of washed blood was aliquoted into 5ml

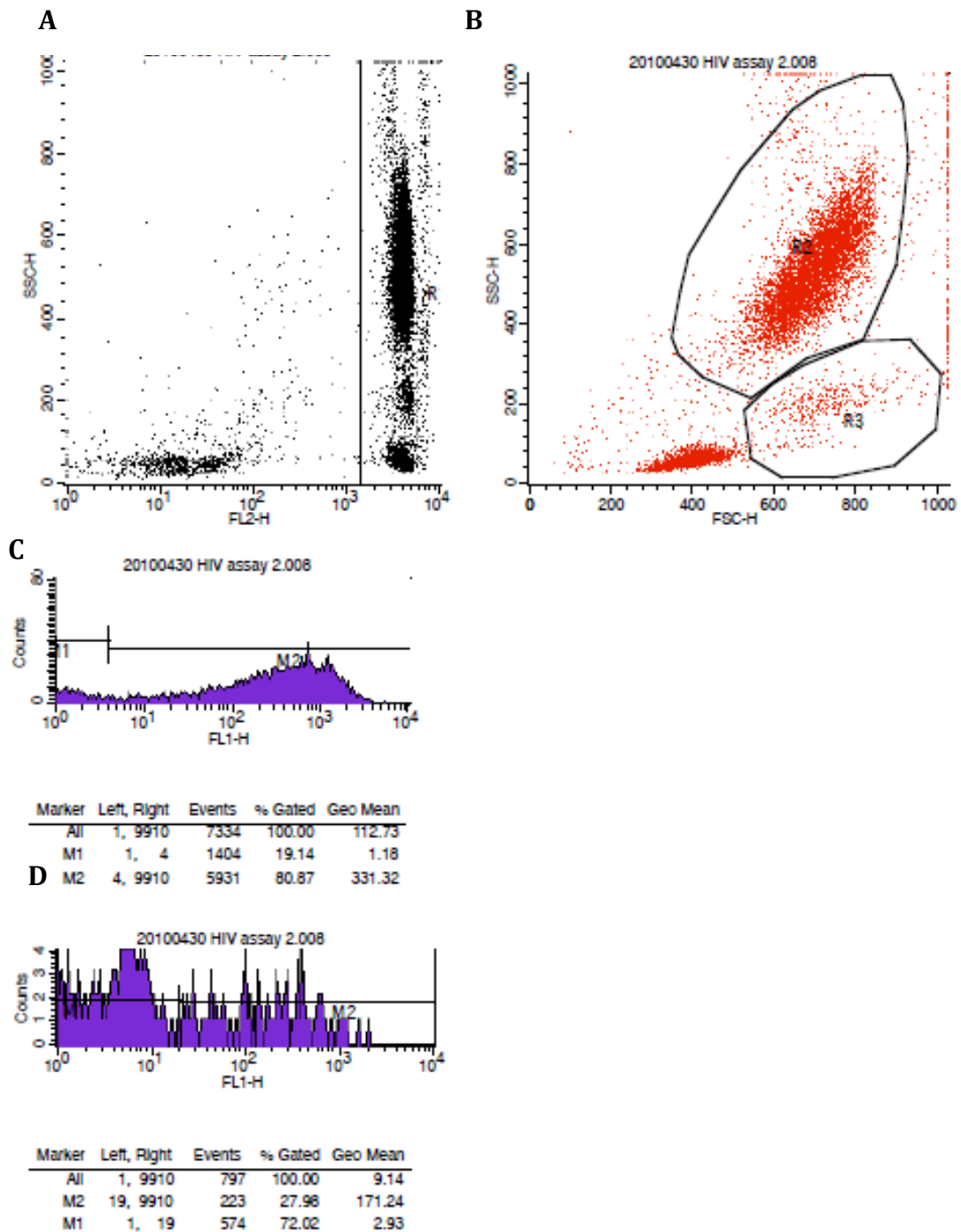


Figure 2.2 Identification of neutrophil and monocyte populations in peripheral blood with flow cytometry.

(A) Dot plots with FL-2 channel gating to exclude non-viable cells. (B) Typical side scatter/forward-scatter dot plots with gates (R2) on neutrophils and (R3) on monocytes. FL-1 histograms showing percentage (M2 % gated) of neutrophils (C) and monocytes (D) which have phagocytosed bacteria.

FACS tubes, and stimulated with 10 µl of opsonised *Salmonella* for 10 min at 37°C in a water bath. 10 µl of dihydrorhodamine-1,2,3 (Orpegen Pharma, Germany) was added to each tube and all tubes were incubated for a further 10 min at 37°C. The dihydrorhodamine is oxidised to rhodamine (which fluoresces) by oxidative burst activity. The amount of fluorescence detected in the FL1 channel represents the oxidative burst activity within the cells following stimulation with *Salmonella*. 1.5 ml 1 x FACS Lysing solution was added to each tube to lyse red cells and samples were vortexed and incubated for 20 min at room temperature in the dark. Samples were then centrifuged at 250 x g, 4°C for 5 min, and the supernatant discarded. The cells were washed twice with 1.5 ml PBS, centrifuged at 250 x g, 4°C for 5 min and supernatant aspirated each time. Following the second wash, 100 µl propidium iodide (20 µg/ml) was added to each tube and the samples were incubated on ice for 10 min before analyzing using a FACS Calibur flow cytometer and Cellquest Pro software. Neutrophils, monocytes and lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC) characteristics. The geometric mean fluorescence intensity in the FL1 channel representing the average oxidative burst activity for each cell type was determined in each sample.

2.2.8 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO QUANTIFY RELATIVE AMOUNT OF ANTI-LPS ANTIBODIES IN SERUM

50 µl of 1 mg/ml Lipopolysaccharide (LPS) purified from *Salmonella* Typhimurium (Alexa) was diluted in 10 ml carbonate coating buffer (0.015 M Na₂CO₃, 0.035 M

NaHCO₃ pH9.6) to give a final concentration of 5 µg/ml LPS. 96-well flat bottom maxisorp plates (Nunc) were incubated overnight at 4°C, with 100 µl of 5 µg/ml LPS per well. The following day, plates were washed three times with 'wash buffer' (PBS 0.05% Tween) to remove any unbound LPS. Wash buffer was made by mixing 1 l PBS with 500 µl Tween (Sigma). Following washing, plates were incubated with 200 µl 'block buffer' (PBS 1% BSA) per well for 1 h at 37°C. Block buffer was made by mixing 100 ml PBS with 1 g Bovine Serum Albumin (BSA) (Sigma). Plates were washed again and 150 µl of a 1:10 dilution of each serum or column fraction in PBS 0.05% Tween 1% BSA was added to the first row of the plate and 100 µl of PBS 0.05% Tween 1% BSA was added to the remaining wells. Three-fold dilutions of each sample were made by transferring 50 µl from the first well of each column to the second well and serially diluting 50 µl into each subsequent well (Figure 2.3). Plates were incubated at 37°C for 1 h before washing three times with PBS 0.05% Tween to remove any unbound antibody. Goat anti-human alkaline-phosphatase conjugated secondary antibodies (Southern Biotech) were diluted 1:2000 in PBS 0.05% Tween 1% BSA and 100µl of secondary antibody (IgG, IgA or IgM) were added to each well. The plates were incubated once more for 1 h at 37°C. Finally, plates were washed three times with PBS 0.05% Tween to remove any unbound secondary antibody and 100 µl of Sigma-fast p-nitrophenyl phosphate substrate was added to each well. The substrate was prepared by dissolving 2 tablets of p-nitrophenyl phosphate (Sigma) in 20 ml distilled water. Plates were incubated again at 37°C and optical density was measured after 60 min using an ELISA plate reader set at 405nm and SoftMax Pro software.

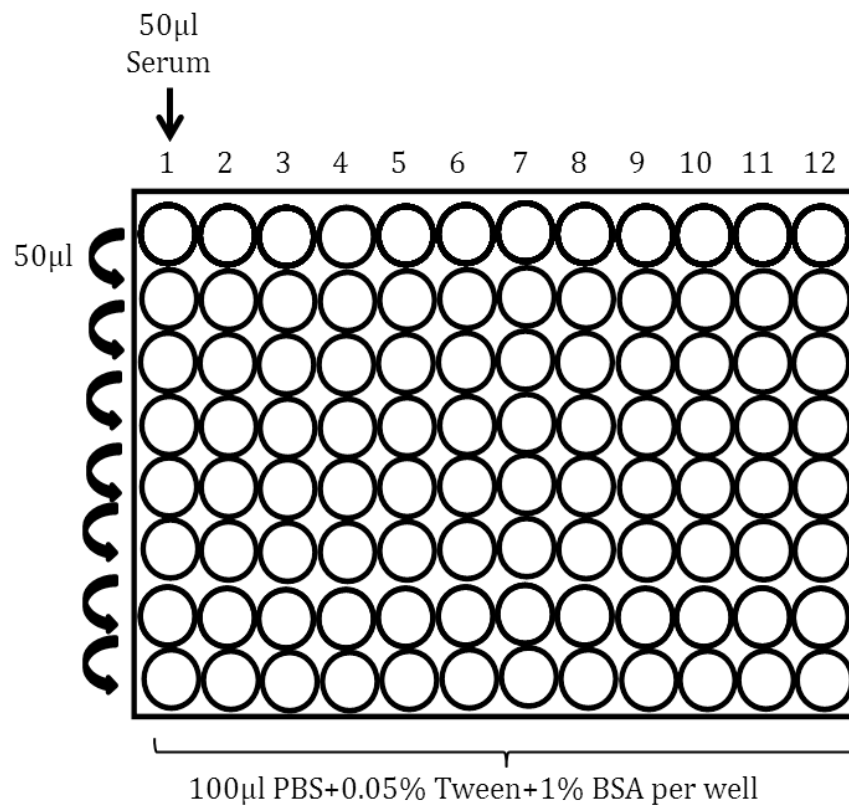


Figure 2.3 Dilution of serum in a 96 well ELISA plate.

Each well of the 96 well plate contained 100 µl PBS+0.05% Tween+1% BSA, and 50 µl of diluted serum was added to the first row of the plate. 50 µl of the solution in each well was transferred to the next row, and this was repeated 8 times and 50 µl discarded from the final row of the plate.

2.2.9 FLOW CYTOMETRIC ANTIBODY ASSAY TO QUANTIFY BINDING OF ANTIBODY TO THE SURFACE OF *SALMONELLAE*

Salmonellae were prepared as described in section 2.2.1.3 and the concentration adjusted to 2×10^9 cfu/ml before fixing with 37% formaldehyde (Sigma) to give a final concentration of 1% formaldehyde. 5 μ l of fixed bacteria were added to 45 μ l of serum diluted 1:10 with PBS or PBS (negative control) in microfuge tubes. After incubating for 20 min at room temperature, 1 ml PBS was added to each tube and the samples were mixed by inversion before centrifuging for 5 min at 3300 x g. The supernatant was discarded and the samples were washed again with 1 ml PBS and centrifuging as before. This time the supernatant was aspirated, leaving a residual volume of 50 μ l. 2 μ l of FITC-conjugated polyclonal rabbit anti-human IgG, IgA or IgM antibody (Sigma) were added to each sample and the tubes were incubated for 20 min in the dark at room temperature. Samples were washed twice more with PBS as before and 200 μ l 1% formaldehyde PBS was added to each sample to fix the bacteria. Samples were analysed by a FACS Calibur flow cytometer (Becton Dickinson) and Cellquest Pro software to acquire and analyse the data. The *Salmonella* population was identified using forward and side scattered light and fluorescence emission in the FL1 channel (Figure 2.4). The geometric mean fluorescence intensity (GMFI) in the FL1 channel was used as the anti-*Salmonella* antibody titre.

To measure complement deposition on the surface of the bacteria, the same assay was used but with 45 μ l undiluted serum instead of a 1:10 dilution of the serum, and with

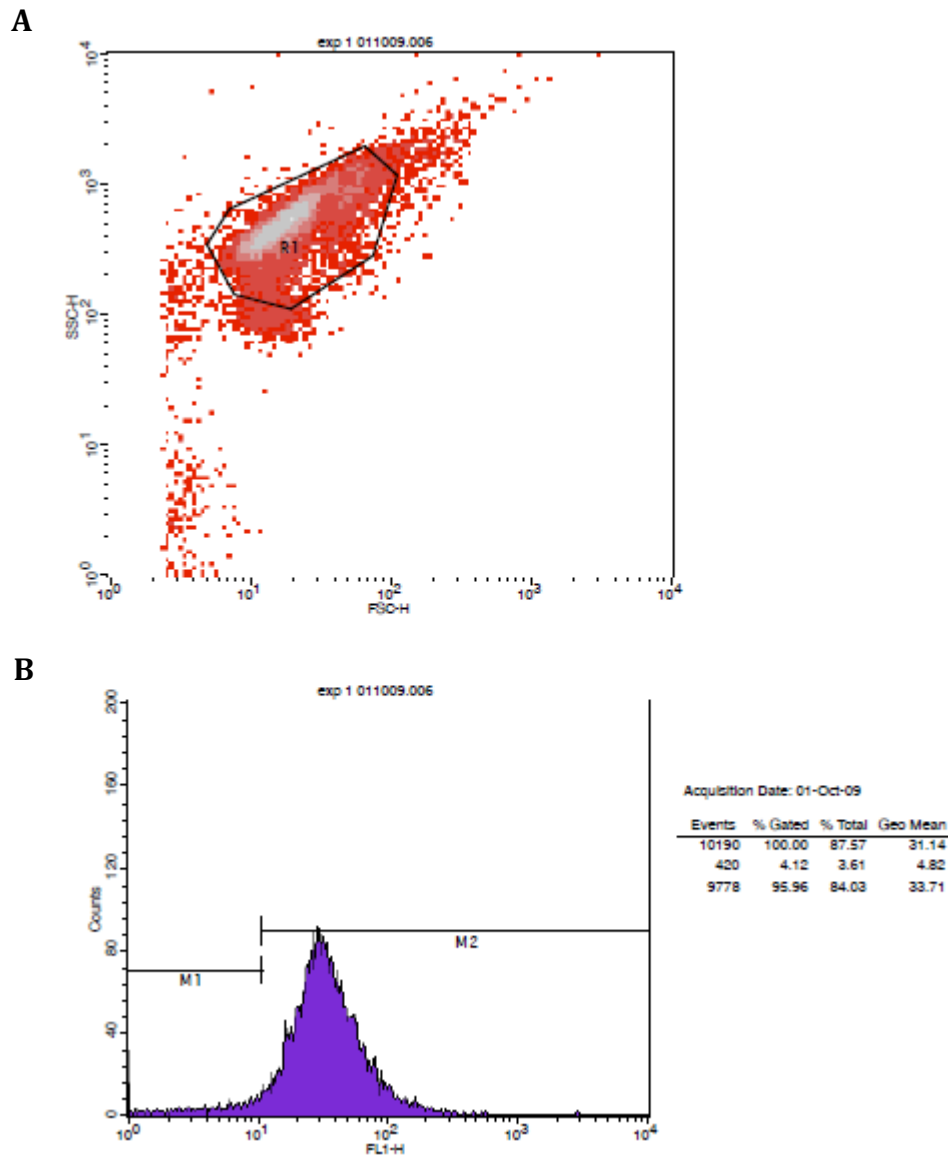


Figure 2.4 Identification of *Salmonellae* population with a flow cytometer.

(A) Density dot plot using Cell Quest Pro software of forward-scattered/side scattered light with the *Salmonella* population detected in gate R1. (B) FL-1 histogram showing fluorescence detected in the FL-1 channel from the FITC emission spectrum. The geometric mean fluorescence intensity (GMFI) in the FL-1 channel is the anti-*Salmonella* antibody titre.

different FITC-conjugated antibodies. To measure C3 deposition a FITC-conjugated anti-C3 antibody (Dako Cytomation) was used, and to measure MAC binding, an anti-C5b-9 antibody in combination with a rabbit-anti-mouse FITC conjugated antibody (Dako Cytomation) was used in place of anti-Ig antibodies.

2.2.10 HAEMOLYTIC ASSAYS

Radial immunodiffusion assays (The Binding Site, UK) were used to determine the alternative and total haemolytic activity of human serum. The assay comprises plates which are composed of chicken erythrocytes (Alternative Pathway Hemolytic Complement kit) or sheep erythrocytes (Total Hemolytic Complement kit). 5 µl of human serum was added to individual wells in the plate and the plates were incubated overnight at 4°C to allow the complement components to diffuse into the agarose. The plates were then incubated at 37°C to allow the activation of complement. For the alternative pathway, the chicken erythrocytes will bind C3b spontaneously formed in the absence of antibody leading to the formation of C3 convertase, which further activates the alternative complement pathway and leads to the haemolysis of the erythrocytes. For the total pathway kit, the sheep erythrocytes are coated with anti-sheep-erythrocyte antibody (haemolysin) which activates the classical complement pathway leading to haemolysis of the erythrocytes. After incubating for 2 h at 37°C, zones of haemolysis are produced around wells proportional to the amount of functional complement in each sample (Figure 2.5). The diameters of these zones were measured and the amount of functional complement activity calculated, using a standard curve of known haemolytic activity.



Figure 2.5 Radial immunodiffusion assay to measure haemolytic complement activity.

Zones of haemolysis are produced around wells containing serum samples. The diameter of the zone is proportional to the amount of functional complement in each sample. If no clear zone is present then complement within the serum sample is not functional.

2.2.11 ABSORPTION OF ANTI-LPS ANTIBODIES FROM HUMAN SERUM USING LPS ATTACHED TO A SOLID MATRIX

50 µl of 1 mg/ml Lipopolysaccharide (LPS) purified from *Salmonella* Typhimurium (Alexa) was diluted in 10 ml carbonate coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃ pH 9.6) to give a final concentration of 5 µg/ml final concentration of LPS. A 96-well flat bottom Maxisorp plate (Nunc) was incubated overnight at 4°C with 100 µl of 5 µg/ml LPS per well. The following day, the plate was washed three times with 'wash buffer' (PBS 0.05% Tween) to remove any unbound LPS. Following washing, 100 µl of serum was added to each well in the first column of the plate and the plate was incubated for 30 min at 4°C. After 30 min, the serum was transferred from the first column to the second column of the well and the plate incubated at 4°C for 30 min again (Figure 2.6). After 12 transfers, the anti-LPS antibody-depleted serum was frozen at -80°C in microfuge tubes.

2.2.12 DEPLETION OF ANTI-SALMONELLA TYPHIMURIUM D23580 ANTIBODIES FROM HUMAN SERUM

Salmonella Typhimurium was cultured as described in section 2.2.1.2. Stationary phase cultures were washed with PBS and re-suspended in PBS to give a final concentration of 1 x 10¹¹ cfu/ml. Re-suspended *Salmonella* and freshly thawed serum were pre-cooled to 4°C. 1 ml of serum was then incubated with 100 µl of *S. Typhimurium* D23580 1 x 10¹¹ cfu/ml for 1 h at 4°C with continuous mixing. The mixture was then centrifuged at 3300 x g, 4°C for 5 min to remove the bacteria, and the supernatant removed by aspiration.

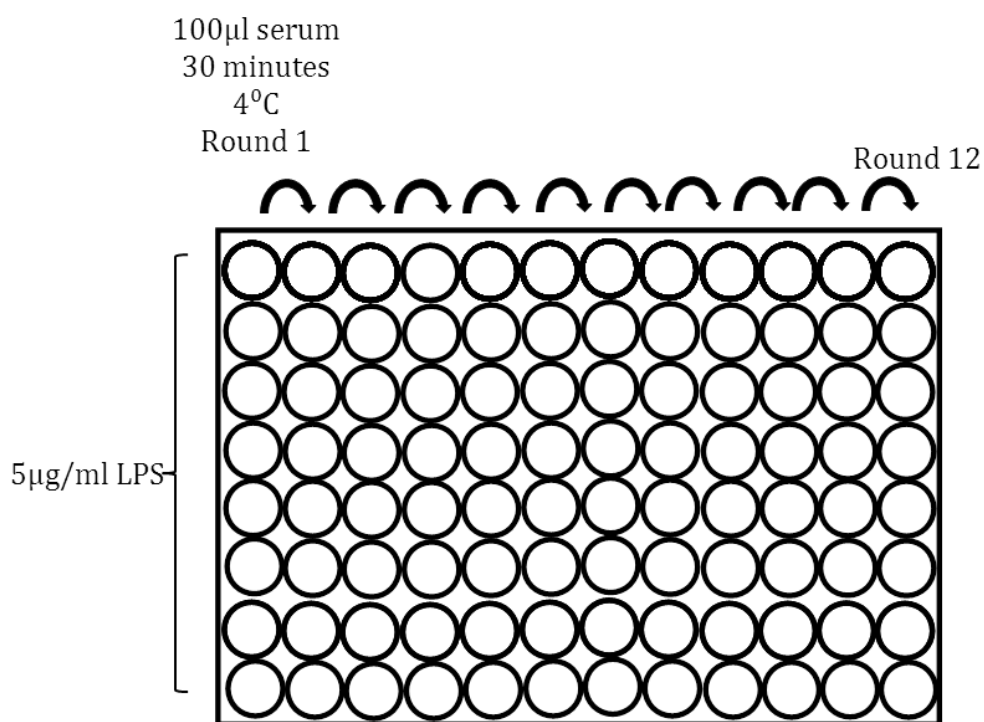


Figure 2.6 Anti-LPS antibody depletion of human serum.

100 µl of serum was added to the first well in a 96-well flat bottom plate which was coated with 5 µg/ml LPS and incubated for 30 min at 4°C and then transferred to the next well (round 1). This was repeated until 12 rounds of incubations and transfers were completed.

100 µl of *S. Typhimurium* D23580 was added to the supernatant and the mixture was incubated again for 1 h at 4°C prior to centrifugation. This step was repeated once more. The final supernatant was removed by aspiration and filtered using a 0.2 µm syringe filter to ensure all bacteria had been removed, and stored at -80°C until required.

2.2.13 AFFINITY PURIFICATION OF ANTI-O-ANTIGEN ANTIBODY FROM HUMAN SERUM USING ACTIVATED O-ANTIGEN LINKED TO NHS-SEPHAROSE RESIN

2.2.13.1 PREPARATION OF O-ANTIGEN FROM LPS

LPS from *S. Typhimurium* D23580, a clinical isolate of NTS, was used as the source of O-antigen (OAg). OAg was purified using a new method developed at Novartis Vaccines Institute for Global Health, Italy (Patent PAT054746). Lipid A was removed by acetic acid hydrolysis of LPS. 100 µl of acetic acid (1% in solution) was added to 10 ml bacterial culture and the solution was heated for 1 h at 100°C, and then centrifuged at 3300 x g overnight. The supernatant was recovered, and neutralized to pH 6 with 1 M sodium hydroxide (Sigma). Absolute ethanol was added to give a final concentration of 80%, causing the OAg to precipitate. The solution was centrifuged again at 3300 x g and the pellet dried overnight using a SpeedVac vacuum centrifuge (Thermo) at room temperature, 500 mtorr. The amount of OAg recovered was quantified using a phenol sulphuric assay.

2.2.13.2 PHENOL SULPHURIC ASSAY

A phenol sulphuric assay is a colorimetric technique used to quantify the amount of sugar present in a solution. It was used to measure the concentration of activated OAg

which had not bound to the NHS-Sepharose resin. 20 mg of glucose (Sigma) was solubilized in 40 ml distilled water to give a concentration of 0.5 mg/ml. 5 g phenol (Sigma) was added to 100 ml distilled water to produce 5% phenol (w/v). Each sample was diluted 1:10 with distilled water in 4 ml glass tubes (Becton Dickinson) to give a final volume of 200 μ l. 200 μ l of 5% phenol solution was added to each tube, followed by 1 ml concentrated sulphuric acid. The samples were left for 10 min then vortexed and incubated for a further 30 min. The absorbance at 490 nm was measured using a spectrophotometer.

2.2.13.3 ACTIVATION OF O-ANTIGEN WITH ADH

Two methods were used to link purified OAg to adipic dihydrazide (ADH). The first method links the NH_2 of the hydrazide group of ADH to the $\text{C}=\text{O}$ of the ketone group of the KDO sugar at the end of the core region of LPS. The second method involves the oxidation of the OAg chain structure at multiple points, resulting in the formation of $\text{C}=\text{O}$ aldehyde groups, prior to activation with ADH, and consequently the linking of more than one ADH molecule to the OAg chain through the formation of an amino link.

2.2.13.4 ACTIVATION OF O-ANTIGEN WITH ADH TO PRODUCE OAGADH

16.7 mg of purified OAg (see 2.2.13.1) was solubilized in 0.42 ml 0.1 M sodium acetate (Sigma) buffer pH 4.5 and 20 mg of ADH and 20 mg sodium cyanoborohydride was added and the mixture heated for 1 h at 30°C. The mixture was purified by desalting against water using a 5 ml HiTrap PD10 desalting column (GE Healthcare) and an ACTA

purifier (GE Healthcare). The reaction product was then dried overnight using a SpeedVac vacuum centrifuge as before and stored at 4°C until required.

2.2.13.5 ACTIVATION OF O-ANTIGEN WITH ADH TO PRODUCE OAGOXADH

16.7 mg of OAg was solubilized in 1.5 ml 0.1 M sodium acetate buffer pH 4.5, and 100 µl 100 mM freshly prepared sodium metaperiodate was added to oxidise the OAg. The mixture was incubated for 2 h at room temperature in the dark, and purified by desalting against water using a 5 ml PD10 HiTrap desalting column (GE Healthcare) and an ACTA purifier (GE Healthcare), prior to drying overnight in a SpeedVac vacuum centrifuge (Thermo), at room temperature, 500 mtorr. This oxidized OAg was then activated with ADH following the same procedure described in section 2.2.13.4 to produce OAgoxADH.

2.2.13.6 IMMOBILIZATION OF ACTIVATED O-ANTIGEN TO THE RESIN OF HITRAP NHS COLUMNS

6.6 mg of activated OAg (OAgADH or OAgoxADH) was dissolved in 1 ml coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl pH 8.3 (Sigma)). Two 1 ml HiTrap NHS-Sepharose columns (GE Healthcare) were washed with 6 ml 1 mM HCl to remove the isopropanol storage solution. The dissolved activated OAg was slowly passed through the column using a 5 ml syringe connected to the column, and the flow through was collected to allow quantification of bound OAg to the column. The columns were incubated overnight at 4°C to allow maximum binding of the NH₂ group of the ADH molecule with the N-

hydroxy-succinamide group of the resin, forming an amido group. Following the incubation, both columns were washed with 6 ml 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 to block and deactivate any sites on the resin which had not bound OAg, followed by 6 ml 0.1M sodium acetate, 0.5 M sodium chloride pH 4 which washes away unbound OAg. All washes were collected and the amount of OAg detected using a phenol sulphuric assay (see 2.2.13.2). This allowed for the quantification of the amount OAg un-linked to the resin and by subtracting the amount of activated OAg lost in the flow through and wash steps from the original amount of OAg loaded onto the column, the amount of OAg linked to the column could be quantified. The column was stored in 0.05 M Na₂HPO₄, 0.1% NaN₃ pH 7 at 4°C until further use.

2.2.13.7 AMMONIUM SULPHATE PRECIPITATION OF SERUM PROTEINS

0.5 g ammonium sulphate (Sigma) was added to 1 ml serum to give a final concentration of 0.5 g/ml and the solution was incubated overnight at 4°C. The following day, the sample was centrifuged at 4°C, 3300 x g for 5 min, and the supernatant removed by aspiration. The precipitate was washed twice with 1 ml 0.5g /ml ammonium sulphate, centrifuging at 3300 x g, 4°C for 5 min and the supernatant removed. The pellet was re-suspended in 1 ml PBS and transferred into pre-soaked dialysis membrane (Spectrum labs) with a molecular weight cut off 6-8 kDa. The precipitate was dialysed against 500 ml PBS for 24 h, with the PBS replaced four times during this period.

2.2.13.8 AFFINITY PURIFICATION OF ANTI-S. TYPHIMURIUM O-ANTIGEN ANTIBODIES FROM HUMAN SERUM

1 ml HiTrap NHS-Sepharose columns (GE Healthcare) with activated OAg linked to the resin (see 2.2.13.6) were washed with 6 ml PBS using a 5 ml syringe connected to the column, to equilibrate the resin. The washes were discarded. 300 µl of antibody precipitate (see ammonium sulphate procedure 2.2.13.7) was applied slowly to the column and the flow through collected. After an overnight incubation at 4°C, the column was washed with 6 ml PBS, followed by 6 ml NaPi 50 mM, NaCl 500 mM pH 7.2, to remove any unbound antibody. All washes were collected. To elute bound antibody, 3 ml 0.1 M glycine 0.1 M NaCl, pH 2.4 was injected into the column and the eluted antibodies collected in 500 µl aliquots which were neutralized immediately with 40 µl 2 M Tris HCl pH 9 (Sigma). All washes and eluates were tested in an ELISA using plates coated with LPS (as described in method in section 2.2.8) and eluates containing anti-LPS antibodies were pooled. The pooled eluates were loaded into pre-soaked and washed dialysis membrane (Spectrum Labs) with a molecular weight cut-off 6-8 kDa. The pooled eluates were dialyzed against 500 ml PBS for 24 h, with the PBS changed four times during this period. After dialysis, the eluates were stored at 4°C in microfuge tubes.

CHAPTER 3.

STABILITY OF COMPLEMENT-MEDIATED BACTERICIDAL ACTIVITY IN HUMAN SERUM AGAINST NONTYPHOIDAL *SALMONELLA*

3.1 INTRODUCTION

The complement system is an important component of the innate immune system. It comprises a set of plasma proteins that facilitate the killing of pathogens (184;185) through the activation of three different pathways which generate a series of effector proteins. The complement fragment C3b can bind to bacteria and act as an opsonin increasing uptake by phagocytic cells (145). Complement can also kill bacteria in a cell-independent manner through formation of the membrane attack complex (186). Previous work in our lab has shown that whole human serum from Africans is able to effect both cell-independent bactericidal killing (50) and cell-mediated killing (151) of African invasive isolates of nontyphoidal *Salmonella*. Both mechanisms require complement and antibody to *Salmonella* (50;151).

Research undertaken throughout this PhD will involve the use of whole human serum in assays to investigate immunity to *Salmonella* Typhimurium. These studies involve the use of sera from African patients and consequently are of limited supply. Blood from these children and adults was taken and sera processed in clinics in Malawi between 2004 and 2008, transported to the UK and stored continuously at -80°C. The assays we use rely on the integrity of endogenous complement in the serum. Therefore care needs

to be taken when processing and manipulating both blood and serum in order to preserve complement function (187) and to minimise the waste of such valuable samples as it has long been known that complement is labile. Antibody molecules are relatively resistant to degradation, heat treatment (188) and can tolerate freeze-thaw cycles. Complement activity though is particularly heat-labile (189;190) and is destroyed by incubating at 56°C for 30 min. Consequently, it is generally advised that serum is promptly separated from clotted blood and stored at -80°C in aliquots to avoid potentially detrimental freeze-thaw cycles. It is also normal practice for serum aliquots to be used only on the day they are thawed and that experiments involving complement are carried out at 4°C.

The stability of complement is also of clinical importance, since while it is normally recommended for functional complement studies that serum be separated as soon as possible following venesection and sent frozen, such instructions are often not adhered to, and it is common for there to be a delay before samples arrive at the laboratory. Hence, the finding of reduced or absent functional complement activity in clinical samples is often due to sub-optimal processing and handling rather than a bona fide complement deficiency.

Many published studies about the stability of complement components have used haemolytic assays and focused on animal serum, in particular guinea pig serum (190). Nevertheless, there are many functional differences in complement between species, including human (191), and findings from these studies may not be valid for use with

human serum. There is very limited published literature about the stability of the human complement components required to induce killing of bacteria in vitro. Therefore, a better understanding of the permissible range of conditions under which serum complement activity is preserved, particularly longevity of complement activity at different temperatures and resistance to freeze-thaw cycles would be very helpful.

3.2 OBJECTIVES

In order to minimise the waste of such rare sera we conducted a study to determine the acceptable conditions for the manipulation of human serum that would not be detrimental to its ability to kill *Salmonella* in our serum bactericidal assay. We determined whether freeze-thaw cycles have a detrimental effect on complement activity. We explored the longevity of complement-dependent bactericidal activity of human serum when stored at different temperatures, in particular whether once thawed, serum can be stored at 4°C and used on consecutive days to minimise the waste of serum. The effect of delayed separation of serum from blood and the relevance in a clinical setting was also investigated.

3.3 RESULTS

3.3.1 HUMAN SERA USED IN THE STUDY CONTAINED *SALMONELLA*-SPECIFIC ANTIBODY

The ability of fresh adult human serum to kill NTS has previously been shown to depend on specific anti-*Salmonella* IgG or IgM antibody activating the classical complement

pathway and the consequent deposition of membrane attack complex on the bacterial surface (50). In order to ascertain that the sera used in the study contained antibody directed against *Salmonella*, we assessed the presence of anti-*Salmonella* antibody targeting *S. Typhimurium* D23580 by flow cytometry. Sera from all four healthy European adult subjects (two male, two female) contained IgG, IgA and IgM classes of antibody capable of binding D23580 (Figure 3.1), indicating that these sera could activate complement deposition on D23580 via the classical complement pathway (50).

3.3.2 SERUM BACTERICIDAL ACTIVITY AGAINST NTS IS RETAINED FOR AT LEAST 35 DAYS WHEN SERA IS STORED AT 4°C AND THREE FREEZE-THAW CYCLES DO NOT HAVE AN EFFECT ON COMPLEMENT STABILITY

In order to assess the effect of freeze-thaw and storage on serum bactericidal activity, serum was optimally prepared to maintain complement integrity. Blood was venesected from four healthy European adults (two male, two female) and left to clot at 4°C for 8 h, rather than 22°C to preserve complement integrity, before separating by centrifugation at 4°C. Aliquots were immediately frozen at -80°C. To determine the effect of freeze-thaw cycles on complement stability, samples underwent zero, one, two or three freeze-thaw cycles, by thawing at 22°C and immediately re-freezing at -80°C. Once thawed on day 1, samples were maintained at 4°C. Serum bactericidal assays against *Salmonella Typhimurium* D23580 were used to monitor complement function, as killing of *S. Typhimurium* is dependent on complement as well as antibody. D23580 is sensitive to antibody-dependent complement-mediated killing, undergoing a one to three log₁₀ reduction in bacterial numbers within 3 h of exposure to serum from healthy adults at

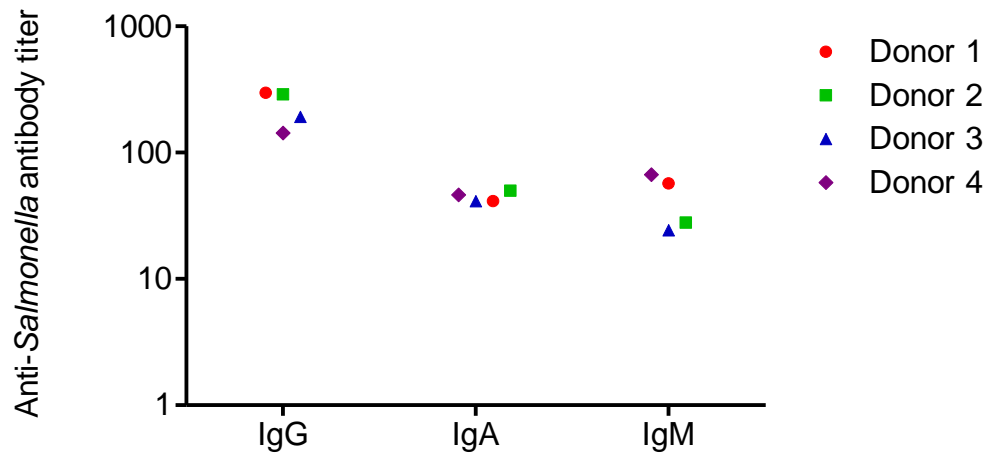


Figure 3.1 Levels of anti-*S. Typhimurium* D23580 IgG, IgA and IgM antibodies in human sera used in the study as assessed by flow cytometry.

Serum from four healthy adults was incubated with *Salmonella* Typhimurium D23580 and the presence of IgG, IgA and IgM classes of antibody targeting *S. Typhimurium* D23580 were measured using flow cytometry. Data are from one experiment. The anti-*Salmonella* antibody titre is the Geometric Mean Fluorescence intensity of fluorescence labelling as measured by flow cytometry using Cell Quest Pro software.

37°C (50). A 1.0 log₁₀ cfu/ml kill is designated as 'normal' kill. To assess the longevity of complement, assays were repeated daily for the first week and then weekly, for a total of 91 days. During the first five weeks of the experiment (up to day 35), each serum killed D23580 by approximately 2.0 log₁₀ cfu/ml (Figure 3.2 A-D). Thereafter, serum from subject 1 was unable to effect a 1.0 log₁₀ cfu/ml kill (Figure 3.2 A), while serum from the remaining three subjects maintained killing for 70 days (Figure 3.2 B-D). We also observed no detectable difference in the ability of serum which had undergone zero, one, two or three freeze-thaw cycles to kill *S. Typhimurium* D23580 (Figure 3.2 A-D).

3.3.3 CLASSICAL AND ALTERNATIVE PATHWAY HAEMOLYTIC COMPLEMENT ACTIVITY IS REDUCED OVER TIME

Invasive strains of *Salmonella* Typhimurium, such as *S. Typhimurium* D23580 can activate complement via the alternative and classical pathways but not the mannose-binding lectin pathway (50). To monitor the classical and alternative pathway complement activity over 91 days, haemolytic complement assays were performed by radial immunodiffusion. Only whole sera which had undergone no freeze-thaw cycles were tested.

A gradual reduction in classical pathway haemolytic complement activity was observed with serum from each subject. Each serum had over 1000 CH100 units/ml on day 0, less than 600 CH100 units/ml by day 28 to 42 and serum from 3 of the subjects had no

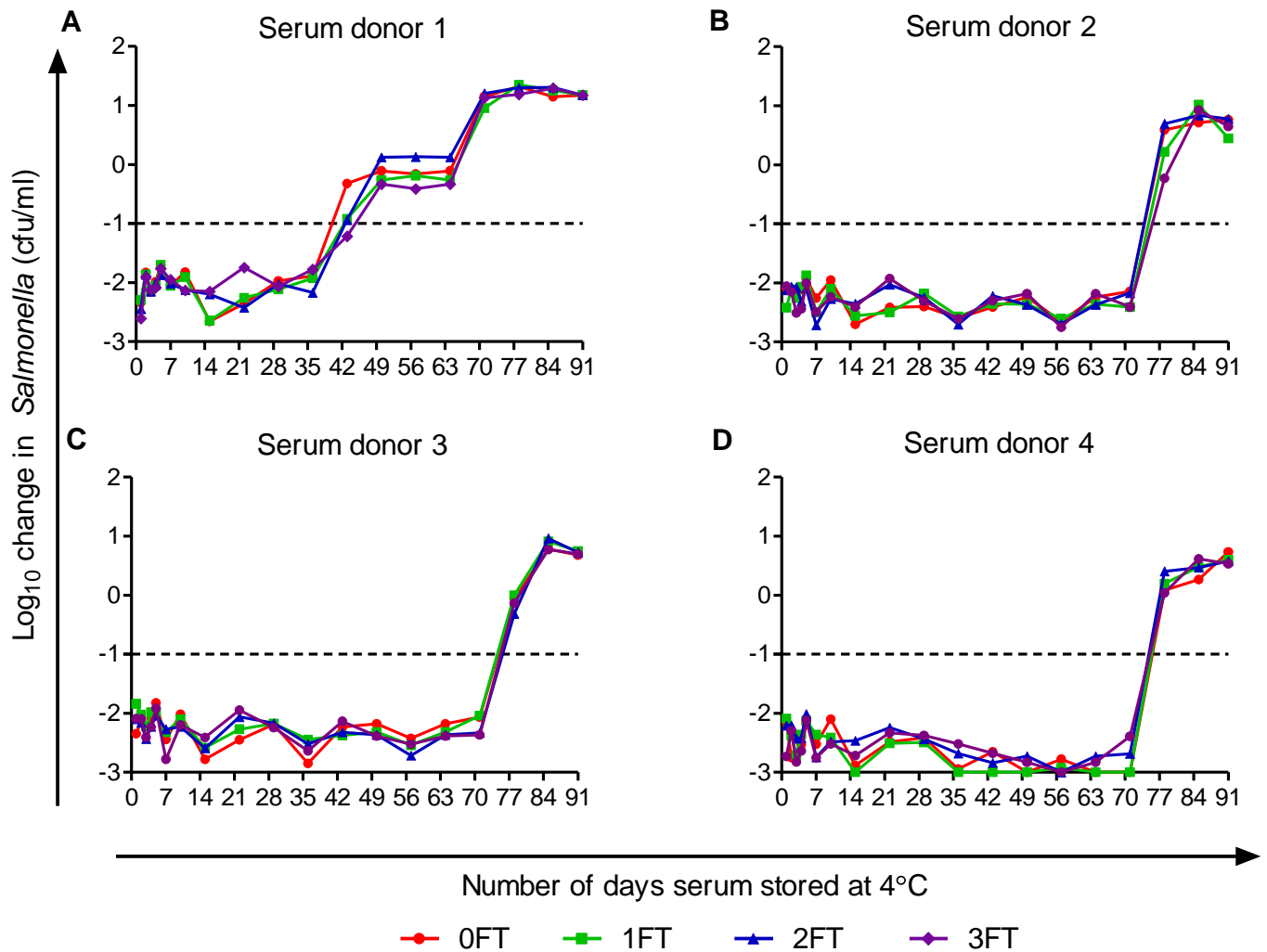


Figure 3.2 Effect of duration of storage of human serum at 4°C and freeze-thaw cycles on its ability to kill *S. Typhimurium* D23580.

Serum from four healthy adults (subjects 1-4 corresponding with panels A.-D.) following one (1 FT), two (2 FT), three (3 FT) or no (0 FT) freeze-thaw cycles, was maintained at 4°C and examined at intervals for ability to kill in the serum bactericidal assay. Normal killing designated as a reduction of 1.0 log_{10} cfu/ml viable bacteria. Numbers of viable *Salmonellae* after 180 min in the assay are plotted against number of days serum kept at 4°C. Negative values show a decrease in viable bacteria compared with initial concentration. Each panel represents data using serum from one adult.

detectable activity on day 49, 63 or 84 (Figure 3.3 A). Alternative pathway haemolytic complement activity was maintained at over 90% normal activity for 42 days in all sera, fell to less than 50% by day 77 and no activity was detectable at 84 or 91 days (Figure 3.3 B).

3.3.4 SERUM HAS REDUCED LONGEVITY OF BACTERICIDAL ACTIVITY AGAINST NTS WHEN STORED AT 22°C AND 37°C

To investigate the effect of increasing the temperature at which serum is stored on its bactericidal activity against *S. Typhimurium*, aliquots of sera from the same four subjects were thawed and maintained at either 22°C (Figure 3.4 A) or 37°C (Figure 3.4 B) post-thawing. Serum bactericidal assays were performed daily using sera stored at 22°C for 15 days. Sera kept at 22°C could effect a 2.0 to 3.0 log₁₀ kill after 180 min for 8 to 10 days after which bactericidal activity fell to less than 1.0 log₁₀ cfu/ml. Sera kept at 37°C were assessed six- to twelve-hourly for bactericidal activity against D23580. At this temperature, bactericidal activity fell more rapidly than at 22°C and was less than 1.0 log₁₀ cfu/ml after 54 h for two sera and after 72 h for the other two sera.

3.3.5 THE PERCENTAGE OF HUMAN SERUM REQUIRED TO KILL NTS INCREASES INCREMENTALLY WITH TIME

The previous experiments were conducted with whole serum with a steady level of killing of *Salmonella* in the bactericidal assay present for a minimum of 35 days, 8 days

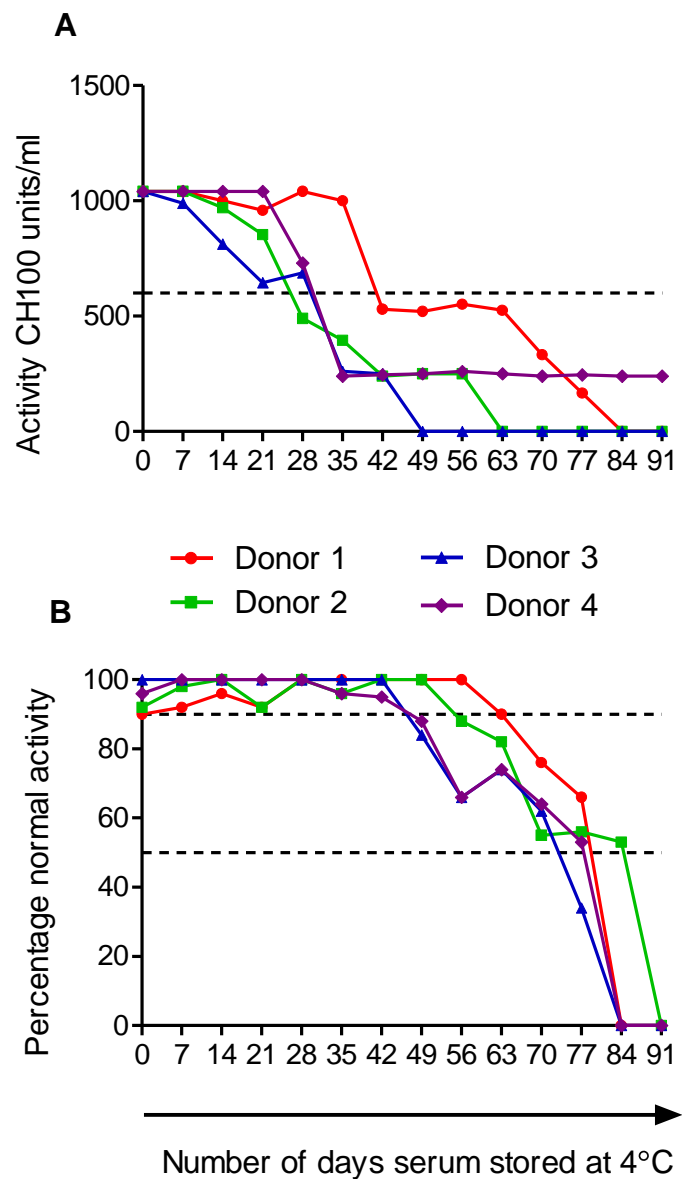


Figure 3.3 Classical and alternative pathway haemolytic complement activity in human serum stored at 4°C for 91 days.

Serum from four healthy adults was stored at 4°C and classical (A) and alternative (B) pathway haemolytic complement activity assessed at intervals using radial immunodiffusion assays. Each panel represents data using serum from four adults.

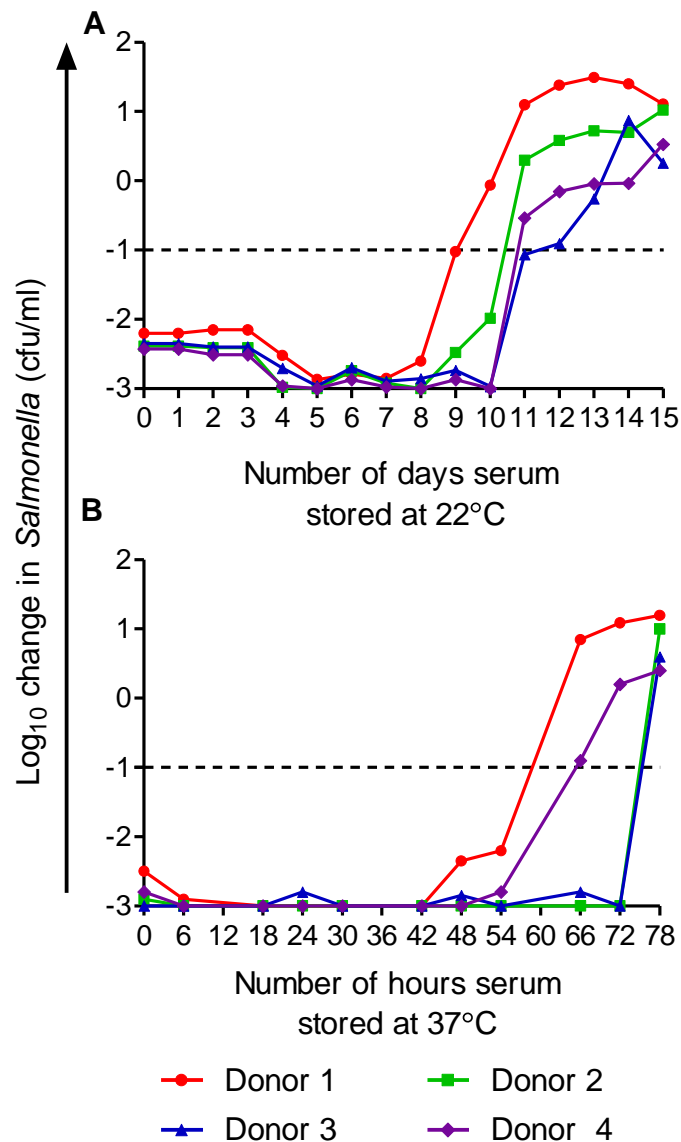


Figure 3.4 Effect of duration of storage of human serum at 22°C and 37°C on ability to kill *S. Typhimurium* D23580.

Serum from the same four adults as in Figure 1 was thawed and maintained at (A) 22°C or (B) 37°C with serum bactericidal assays performed daily (for samples at 22°C) or six- to twelve-hourly (for samples at 37°C) using 10⁶ cfu/ml bacteria. Viable bacteria at 180 min in each serum bactericidal assay are plotted against duration of serum storage. Each panel represents data using serum from four adults.

or 42 hours, dependent on storage temperature. In order to detect whether during this time there is any degradation of bactericidal potential within the sera, we repeated the experiment using serum from two of the four subjects stored at room temperature, whilst assessing the ability of different dilutions of serum to kill *Salmonella*. When first thawed serum concentrations of 20% or above are required to achieve a 1.0 log₁₀ cfu/ml kill of *Salmonella* at 180 min in the serum bactericidal assay (Figure 3.5 A - B).

When subsequently kept at 22°C, the ability of the diluted sera to effect a normal kill decreased more rapidly than with whole serum. The most dilute sera lost the ability to kill first: 20% sera was unable to effect normal killing after one to three days, 30% serum after three to four days, 40% serum after four to five days and 50% after five to six days (Figure 3.5 C and D).

3.3.6 DELAYED SEPARATION OF SERUM FROM CLOTTED HUMAN BLOOD FOR UP TO FOUR DAYS POST-VENESECTION DOES NOT REDUCE ABILITY TO KILL NTS

Delayed separation of serum from blood often occurs due to delays in transportation of blood samples from the clinic to the laboratory. To investigate the effect of up to four days delay in separation of serum from blood on *Salmonella* killing, we performed the serum bactericidal assay using blood kept at either 4°C (Figure 3.6 A, C, E, G) or 22°C (Fig 3.6 B, D, F, H) post-venesection that was either separated on the day of venesection or after one to four days. Assays were performed on all samples a total of four days post-venesection and compared with the killing activity of the respective freshly-

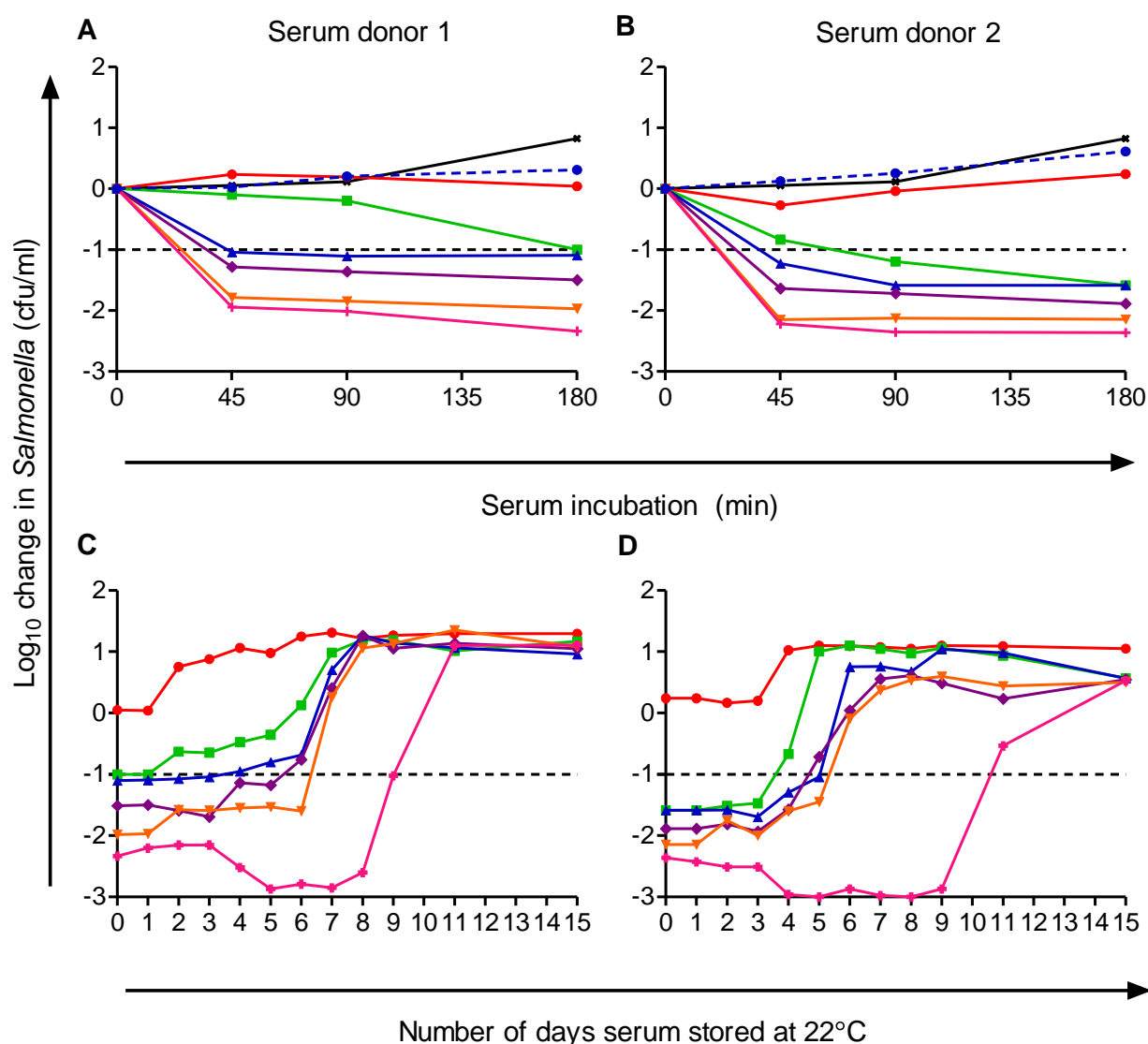


Figure 3.5 Percentage of human serum stored at 22°C required to kill *S. Typhimurium* D23580 and anti-*S. Typhimurium* antibody levels in study sera.

Killing of bacteria by different percentages of freshly thawed serum from (A) subject 1 and (B) subject 2 at 45, 90 and 180 min in the serum bactericidal assay using 10^6 cfu/ml *S. Typhimurium* D23580: 10% (red circles), 20% (green squares), 30% (blue triangles), 40% (purple diamonds) and 50% (orange inverted triangles) serum diluted with PBS, 100% PBS (black crosses) and 100% fresh serum (pink vertical crosses). Initial concentration of bacteria 10^6 cfu/ml with numbers of viable bacteria plotted against duration of assay. Killing of bacteria by different percentages of serum from (C) subject 1 and (D) subject 2 at 180 min following storage at 22°C. Change in viable count (cfu/ml) plotted against duration of time serum stored at 22°C. Each panel represents data using serum from one adult.

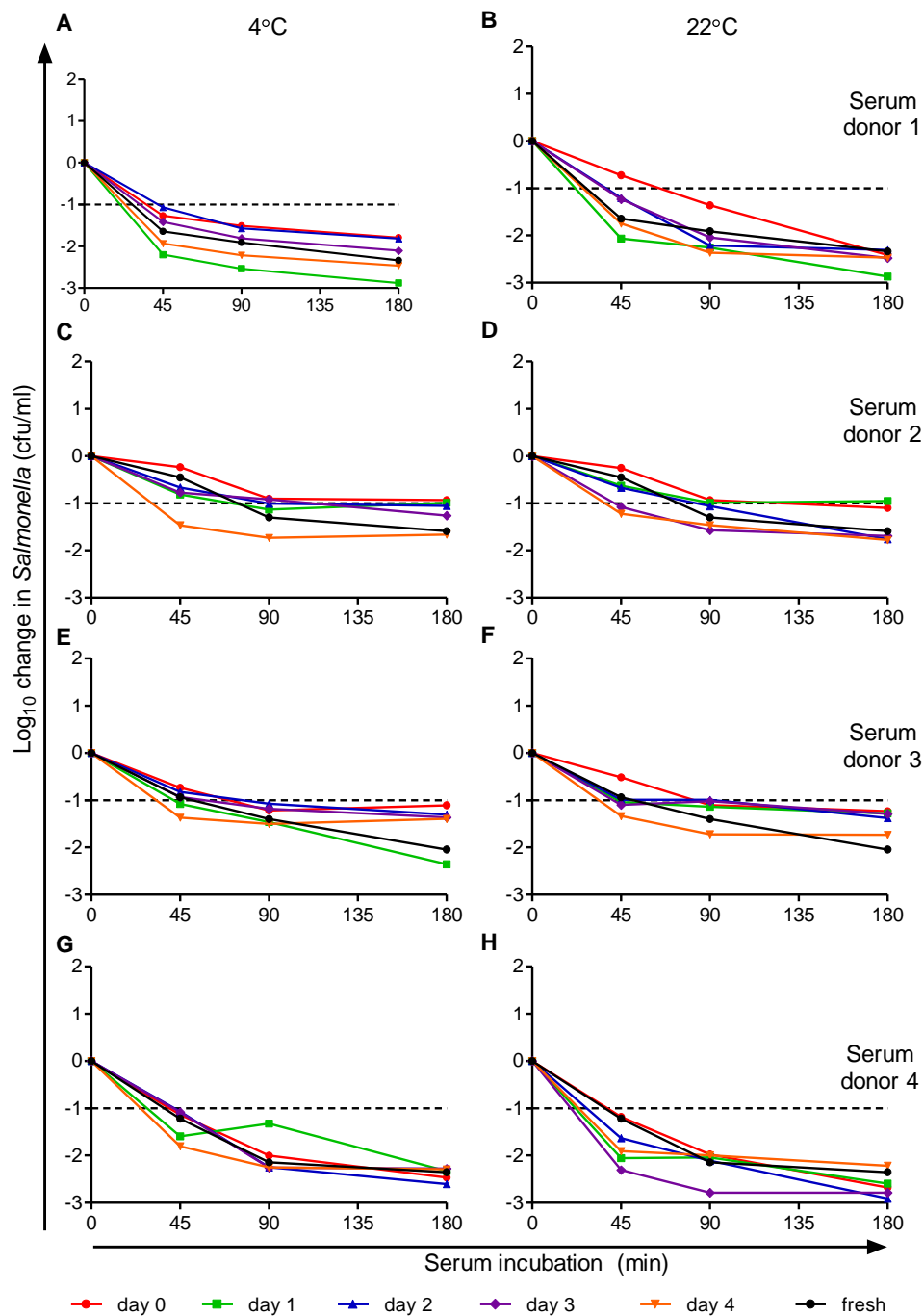


Figure 3.6 Effect of delayed separation of serum from clotted human blood on ability to kill *S. Typhimurium* D23580.

Blood from four healthy adults (subjects 1-4 corresponding with panels A.-B.,C.-D., E.-F. and G.-H. respectively) was kept at 4°C (A., C., E., G.) or 22°C (B., D., F., H.) post-venesection and serum separated the same day (day 0) or after one (day 1), two (day 2), three (day 3), or four (day 4) days after venesection. Separated sera were then maintained at 4°C without freezing for a total of four days post venesection before being compared to freshly thawed serum (fresh) in the serum bactericidal assays. Changes in cfu/ml are plotted against the duration of assay. Each panel represents data using serum from one adult.

thawed serum. There was no significant difference in the bactericidal activity of the serum after 1, 2, 3 or 4 days delay in separation from whole blood at any of the three assay time points (45, 90, 180 min), regardless of whether the blood had been stored at 4°C (ANOVA $p=0.09$ to 0.74) or 22°C (ANOVA $p=0.19$ to 0.99).

3.4 DISCUSSION

Research undertaken throughout this PhD involves the use of a limited supply of sera from African patients to investigate immunity to *Salmonella* Typhimurium. To minimise the waste of such valuable serum we needed to establish the parameters for the optimal acceptable handling and storage of these samples as complement is known to be labile. Much of the available scientific literature describing experimental studies on complement activity date from the first half of the 20th century. Most of these data relate to studies of animal, rather than human complement, and use haemolytic complement assays to assess complement function. These assays may not reflect the bactericidal activity against *Salmonella* or bacteria in general, which usually depend on the combined action of antibody and complement.

A common assumption is that frozen aliquots of serum should be used on the day of thawing when required for bactericidal assays. Our findings surprisingly indicate that this is not the case, since thawed serum stored at 4°C effects normal levels of killing of *Salmonella* for 35 days. For three of the four sera, killing was still obtained after 70 days at 4°C. Our group have previously shown that classical pathway of complement is

required for bactericidal activity against invasive African isolates of *Salmonella* (50). These current findings are consistent with only a small level of classical pathway activity being required for this activity, a level that can be undetectable by haemolytic radial immunodiffusion assay.

Mechanistically, these data are compatible with the hypothesis that once the classical pathway has initiated C3b deposition on *Salmonella*, this is sufficient for the deployment of the alternative pathway that then becomes the critical determinant of bactericidal activity. It is surprising that serum from subject 1 had only half the duration of bactericidal activity of the other three sera when stored at 4°C. Our data do not provide an explanation for this, since longevity of classical and alternative pathway haemolytic complement activity for this serum was no less than that for the other three sera. The difference in longevity of bactericidal activity between this serum and the others was less apparent at 22°C and 37°C.

Functional complement activity was retained in serum stored at 22°C for at least 9 days and 66 hours when stored at 37°C confirming that the longevity of bactericidal activity of human serum against *Salmonella* is reduced with increased storage temperature post-thawing. This finding has also been described using haemolytic assays (189;190). The experiments using diluted human serum support the theory of complement degradation over time. 20% fresh serum is able to kill *Salmonella*, but when stored at 22°C and tested over a 15 day period, the percentage of fresh serum required to induce

the same level of killing increases to 50%, suggesting that a higher percentage of serum is required to provide the same amount of functional complement. Furthermore, previous work by our group shows that when serum is heated at 56°C for 30 min, all complement is destroyed and serum is no longer able to kill *Salmonella* (50). Supplementing this heat-inactivated serum with fresh serum restored killing of *Salmonella* proving that in this assay, complement, but not antibody, is rate limiting (50).

Given the accepted view that freeze-thaw cycles are to be avoided in the context of preservation of complement function (187), then perhaps the most surprising finding of the study was the lack of any discernible effect on bactericidal activity of up to three freeze-thaw cycles. Consistent with this finding, a report from 1927 found that guinea pig serum could be subjected to twelve freeze-thaw cycles without an appreciable decrease of haemolytic activity (188). Further experiments are required though to determine exactly how many freeze-thaw cycles human serum can endure without loss of bactericidal activity.

The clinical and diagnostic implications of this study are important. There can often be a delay between blood being taken in clinics and arriving in the laboratory for testing, making it difficult to decide whether impaired complement function is due to an infection or poor sample handling. Our observation of no difference in complement integrity whether serum is separated immediately from clotted blood or up to 5 days

post-venesection, and stored at either 4°C or 22°C during this time, provides evidence to suggest that any difference with complement function is likely to be due to a defect such as an infection or disease rather than poor sample handling.

One of the limitations of this study is that blood and serum handling was conducted under optimal conditions. Different findings could be expected if for example, frozen serum had been stored at temperatures higher than -80°C, or if during freeze-thaw cycles, serum had been left thawed for extended periods of time before refreezing. Furthermore, only sera from four subjects were tested in this study, which was due to the large number of assays required to determine the conditions for serum manipulation. Killing of *Salmonella* by serum from three donors was maintained over a longer period of time when stored at 4°C, confirming that the longevity of complement function varies between individuals. This may be due, for example, to polymorphisms of the alleles encoding C4. Also, all donors tested in this study were healthy, and the stability of complement function may be reduced in patients with hepatitis C infection or autoimmune diseases causing complement-consumption. Ideally, serum for each new donor should be tested in an established assay system prior to using it routinely.

The practical implications of this study are that if thawed human serum is to be used over an extended number of days, it is important that it is kept refrigerated. This will help to minimise the waste of serum which has been thawed. It is also important to note that human complement does not necessarily behave the same as animal complement

in bactericidal assays (191) and these findings may not apply to animal serum. The findings of the study indicate that the current guidelines concerning manipulation and storage of serum to preserve complement integrity and function leave a large margin for safety. However, it must be noted that the disparity in the storage conditions required for maintenance of haemolytic activity against *Salmonellae* and for complement haemolytic assays may be due to the presence of complement inactivators on mammalian red blood cell surfaces necessitating much higher levels of complement activation for mammalian red blood cell haemolysis to occur. This makes mammalian red blood cell haemolytic assays very sensitive laboratory indicators of complement deficiency. However, for the purposes of bacterial killing, use of the specimen handling conditions they mandate may be unnecessarily stringent.

3.5 CONCLUSIONS

The aim of the present study was to evaluate the conditions under which human serum can be stored and manipulated while maintaining complement integrity, in order to minimise the waste of precious African serum samples. Surprisingly, serum bactericidal activity against *Salmonella* was maintained for a minimum of 35 days when stored at 4°C, suggesting that once thawed serum stored at 4°C can be used over a number of days. The longevity of bactericidal activity was reduced when serum was stored at higher temperatures. Using diluted whole serum confirmed that complement degradation occurs over time. Our results also showed that complement is more robust than expected and that serum can undergo up to three freeze-thaw cycles with no

detrimental effect on bactericidal activity. A delay in the separation of serum for up to four days from clotted blood stored at 4°C or 22°C did not affect bactericidal activity which is useful in a clinical setting. These findings indicate that the current guidelines concerning manipulation and storage of human serum to preserve complement integrity and function leave a large margin for safety with regards to bactericidal activity against *Salmonella*.

CHAPTER 4.

IDENTIFICATION OF AN EXOGENOUS COMPLEMENT SOURCE FOR KILLING OF *S. TYPHIMURIUM* BY BLOOD CELLS

4.1 INTRODUCTION

In order to develop effective vaccines against NTS, an improved understanding of the relative contributions of human antibodies against *Salmonella* antigens needs to be established. Our blood cell killing assay provides an in vitro model of killing of NTS by peripheral blood cells but uses whole human serum as an opsonin, thus making it difficult to identify the role of antibodies against particular targets. Therefore, to help identify the role of specific opsonic antibodies in immunity to NTS, we needed to dissect this assay and identify an exogenous complement source which could be used in conjunction with antibodies, or specific purified antibody, to opsonise *Salmonella* and induce killing by blood cells.

A suitable complement source must not contain antibodies to *S. Typhimurium*. Since we have found that sera from all healthy adults contain naturally acquired antibody to invasive strains of NTS, regardless of exposure to these specific isolates, then sera from healthy adults are not a suitable source. Serum from young children aged 3 months to 2 years often lack specific anti-*Salmonella* antibody (50) hence the peak of NTS bacteraemia in this population, but it is unethical to take blood from these children to act as a source of complement in experiments. We have obtained serum from patients

with primary antibody deficiencies such as common variable immunodeficiency and X-linked agammaglobulinaemia prior to commencement of antibody replacement therapy, from the Department of Immunology, Addenbrooke's Hospital, Cambridge, but these are of limited supply as patients usually begin therapy shortly after diagnosis.

Due to the difficulties in obtaining human serum which is deficient in antibody, many investigators use baby rabbit serum (BRS), which is commercially available, as an alternative exogenous complement source. The World Health Organization recommends the use of baby rabbit serum from 3-4 week old rabbits as a source of exogenous complement in assessing human bactericidal responses to meningococcal polysaccharide vaccines (192-195). BRS has also been used to assess bactericidal activity against *Salmonella* Typhi (196). However, for meningococcal strains, it was found that the bactericidal antibody titre increased if BRS rather than human serum was used as the complement source (197;198), suggesting that differences in complement from different species is an issue. Therefore, if human complement is required, then one approach is to absorb antibodies to *S. Typhimurium* from human serum, by incubating serum with whole bacteria.

4.2 OBJECTIVES

We tested various sources of commercial BRS and depleted human serum of *Salmonella* antibodies to identify a complement source, which is not toxic to *Salmonella* in the absence of antibody, and when antibody is added can effectively opsonize *Salmonellae*

to initiate killing of NTS by peripheral blood cells. The percentage of complement and antibody required to induce cell killing and bactericidal killing was compared.

4.3 RESULTS

4.3.1 OPSONIC ACTIVITY OF DILUTED HUMAN SERUM IS REDUCED COMPARED TO WHOLE SERUM

To determine if diluted human serum can kill *S. Typhimurium* in our blood cell killing assay, which measures the opsonic capacity of antibody and complement to induce killing by peripheral human blood cells, we opsonised *S. Typhimurium* with whole or diluted serum from 2 healthy donors which were known to contain anti-*S. Typhimurium* antibody (Figure 4.2) and measured the clearance from the blood after 45, 90 and 180 min. Opsonisation with whole serum from each donor resulted in a 2.0 log₁₀ kill of *Salmonella* after 180 min (Figure 4.1 A, B). When the sera were diluted with PBS, the highest dilution which could kill *S. Typhimurium* D23580 was 1:2. Dilutions of the sera to 1:4 and further resulted in bacterial growth. Killing in this assay is complement-dependent as heat-inactivation of the serum at 56°C for 30 min removed any killing capacity of the sera (Figure 4.1 A, B). This result highlights that the opsonic capacity of human serum is concentration-dependent and that as the serum is diluted, the ability to induce peripheral blood cell killing is titrated out, suggesting that an exogenous complement source is required when diluted human serum is used as the antibody source. Previous work by our group suggests that killing is limited by

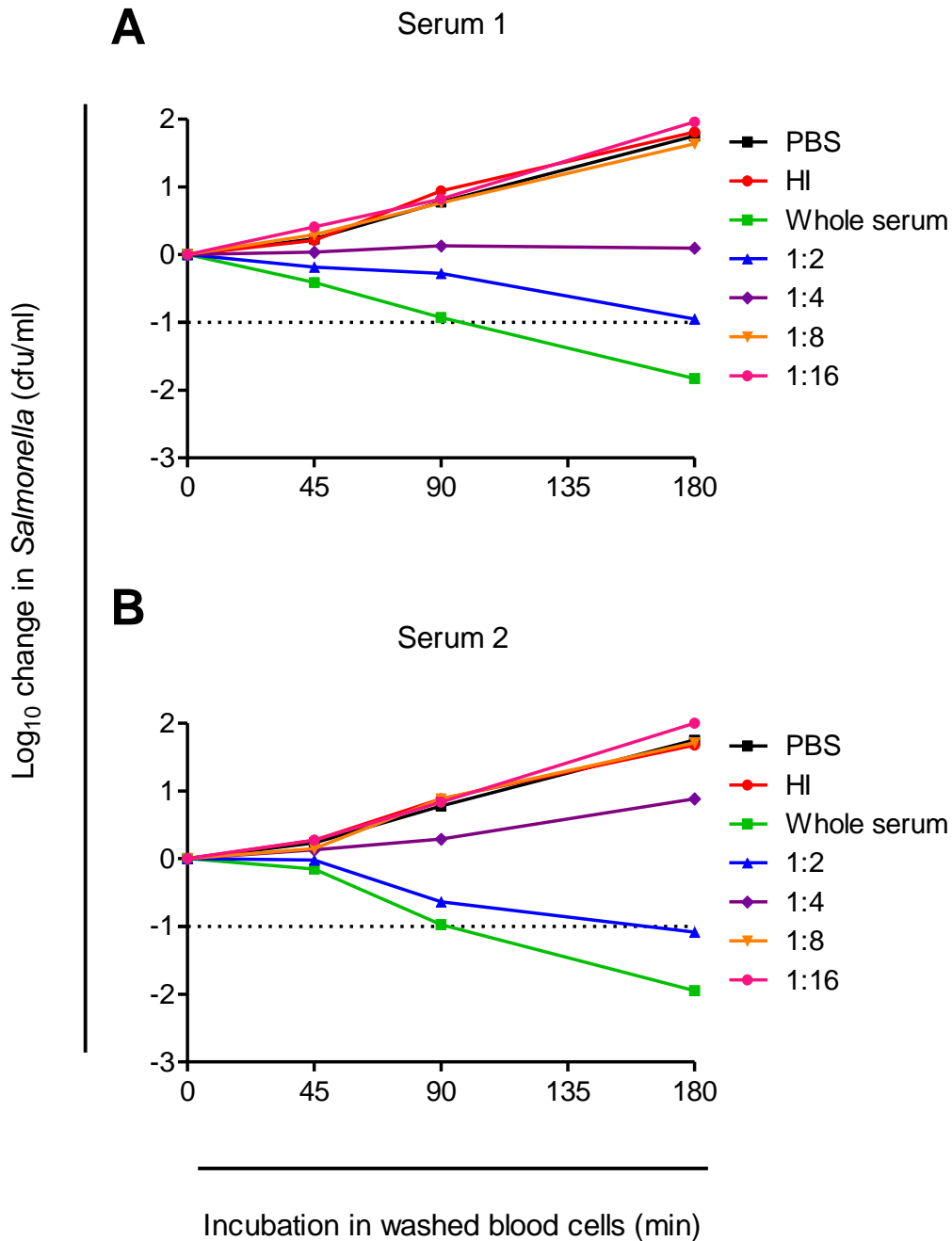


Figure 4.1 Blood cell killing of *S. Typhimurium* D23580 opsonised with diluted human serum.

The effect of opsonisation with diluted human serum on killing by peripheral washed blood cells was measured by opsonising *S. Typhimurium* D23580 with PBS (negative control), heat-inactivated serum (HI), fresh whole serum (whole serum) or serum diluted with PBS (1:2, 1:4, 1:8, 1:16) from 2 healthy adults (A and B). Opsonised *Salmonellae* were incubated with washed adult blood cells and viable bacteria counted after 45, 90 and 180 min. Initial concentration of *Salmonella* is 1×10^5 cfu/ml, with negative results showing a decrease in viable *Salmonellae* compared with the initial starting concentration. Each panel represents data using serum from one healthy adult.

complement as when diluted human serum was supplemented with heat-inactivated human serum killing was not enhanced (50).

4.3.2 BRS DOES NOT CONTAIN ANTIBODIES TO *S. TYPHIMURIUM* D23580

Commercial BRS is often used as an exogenous complement source in serum bactericidal assays (192;196). We first verified that BRS from AbD Serotec or Pelfreez did not contain antibodies to *S. Typhimurium* D23580, using our FACS-based antibody assay (Figure 4.2). No IgG, IgA or IgM anti-*Salmonella* Typhimurium antibodies were detected in either source of BRS, but, both human sera used in the first assay (and for subsequent assays as an antibody source) contained anti-*Salmonella* IgG, IgA and IgM antibodies.

4.3.3 BRS IS TOXIC TO *S. TYPHIMURIUM* D23580 AT HIGH CONCENTRATIONS

We next measured the toxicity levels of BRS to *S. Typhimurium* D23580 in our blood cell killing assay. *S. Typhimurium* D23580 was opsonised with 100%, 75%, 50%, 25% BRS from AbD Serotec and Pelfreez and PBS, and incubated with washed human peripheral blood cells, with viable *Salmonella* numbers determined after 45, 90 and 180 min. *Salmonellae* opsonised with 100% BRS from AbD Serotec and Pelfreez underwent -1.5 log₁₀ kill (Figure 4.3 A) and -2.5 log₁₀ kill (Figure 4.3 B) respectively suggesting that at high concentrations, BRS is toxic to *Salmonella*. Toxicity was diminished when BRS

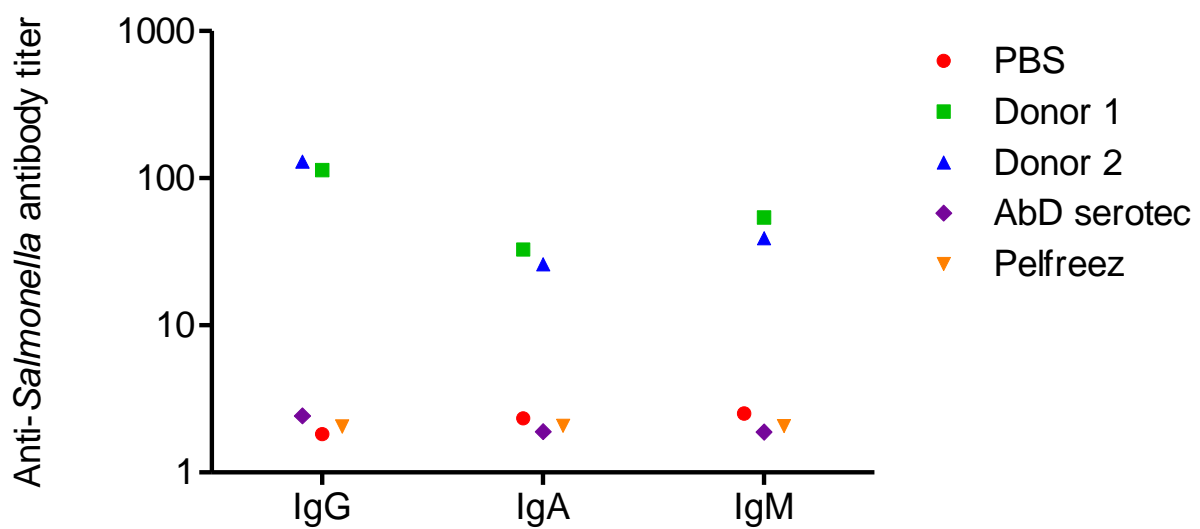


Figure 4.2 Anti- *Salmonella* Typhimurium D23580 antibody levels in BRS and human serum.

Anti-*S. Typhimurium* D23580 IgG, IgA and IgM deposition on *S. Typhimurium* D23580 in BRS from AbD Serotec or Pelfreez and human serum (Donor 1 and Donor 2). PBS included as a negative control. Data are from one experiment.

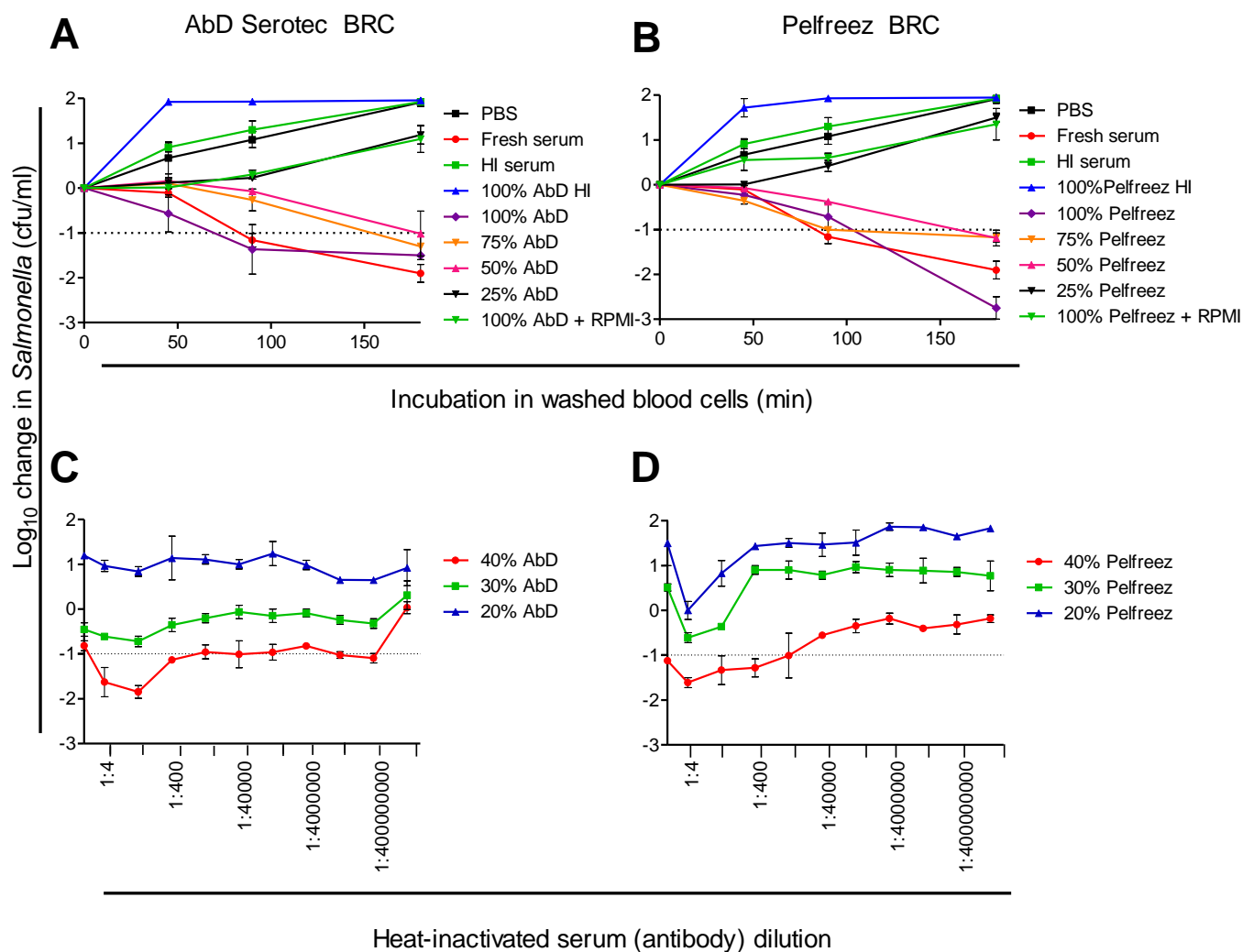


Figure 4.3 Killing of *S. Typhimurium* D23580 opsonised with BRS alone or supplemented with antibody.

Killing of *S. Typhimurium* D23580 opsonised with 100%, 75%, 50% or 25% fresh BRS from AbD Serotec (A) or Pelfreez (B) compared to opsonisation with PBS, fresh human serum (fresh serum), heat-inactivated human serum (HI serum), heat-inactivated BRS (100% AbD HI / 100% Pelfreez HI) by washed peripheral blood cells or by RPMI (100% AbD +RPMI, 100% Pelfreez+RPMI). Viable counts determined after 45, 90 and 180 min. 40%, 30% and 20% of fresh BRS from AbD Serotec (C) and Pelfreez (D) were supplemented with diluted heat-inactivated human serum (10 fold dilution range starting at 1:4) and used to opsonise *S. Typhimurium* D23580 prior to incubating with adult washed blood cells. Change in *Salmonella* concentration after 180 min is plotted against the antibody dilution used to opsonise *Salmonellae*. Initial concentration of *Salmonella* is 1×10^5 cfu/ml, with negative results showing a decrease in viable *Salmonellae* compared with the initial starting concentration. Data represent means \pm SD of 3 experiments.

was heat-inactivated or opsonised *Salmonellae* were incubated in RPMI rather than in washed blood cell suspensions. Opsonisation with 75% BRS and 50% BRS from both sources was also toxic to *S. Typhimurium* effecting a 1 log₁₀ kill in the absence of antibody, but opsonisation with 25% BRS 75% PBS was not toxic, with 1 log₁₀ growth observed after 180 min (Figure 4.3 A and B).

We investigated whether killing of *Salmonellae* opsonised with BRS could be enhanced through the addition of exogenous antibody. Diluted heat-inactivated human serum from donor 1, known to contain anti-*Salmonella* antibody (Figure 4.2), was used as the antibody source. Since opsonisation with 50% BRS alone was toxic, but 25% was not, we tested dilutions of BRS below 50%. Killing of *Salmonella* opsonised with 40% BRS from AbD Serotec and antibody dilutions of 1:4 and 1:40 was increased compared to opsonisation with 40% BRS alone, but 40% BRS also effected a kill in the absence of antibody (Figure 4.3 C). Opsonisation with 30% BRS effected a 0.5 log₁₀ kill and the addition of exogenous antibody, even at a dilution of 1:4, did not enhance killing. Although 20% BRS was not toxic, killing was not increased further when supplemented with antibody.

When the assays were repeated with BRS from Pelfreez (Figure 4.3 D), 40% BRS induced a 1 log₁₀ kill in the absence of antibody, which was increased to 1.5 log₁₀ kill when antibody was added at a dilution of 1:4, but diminished as antibody was diluted further. Opsonisation with 30% BRS was not able to kill *Salmonella*, and a 0.5 log₁₀ kill

was observed when heat-inactivated serum at a dilution of 1:4 and 1:40 was added and decreased as the heat-inactivated serum was diluted further. 20% BRS was not toxic, but no killing of *Salmonella* colonies was observed when supplemented with antibody.

These results suggest that BRS from AbD Serotec or Pelfreez are not suitable complement sources for use in blood cell killing assays since they are toxic to *Salmonellae* at high concentrations, and when used at low concentrations and supplemented with diluted human heat-inactivated serum, killing of *S. Typhimurium* D23580 is not enhanced. Therefore, an alternative complement source is required.

4.3.4 FUNCTIONAL HUMAN COMPLEMENT REMAINS IN SERUM AFTER PRE-ABSORPTION OF ANTI- *S. TYPHIMURIUM* D23580 ANTIBODIES

An alternative complement source to BRS is to pre-absorb serovar-specific antibody from human serum by incubating serum with high concentrations of whole bacteria. We pre-absorbed antibodies to *S. Typhimurium* D23580 from serum taken from a healthy adult. After 3 rounds of incubations with bacteria, the anti-*S. Typhimurium* D23580 IgG, IgA and IgM titres, as assessed by flow cytometry, had decreased confirming that the majority of antibody had been depleted from the serum, providing a human complement source (Figure 4.4 A).

One potential caveat with this antibody depletion process is the loss of complement activity, since complement can be deposited on the bacteria during the incubations. To

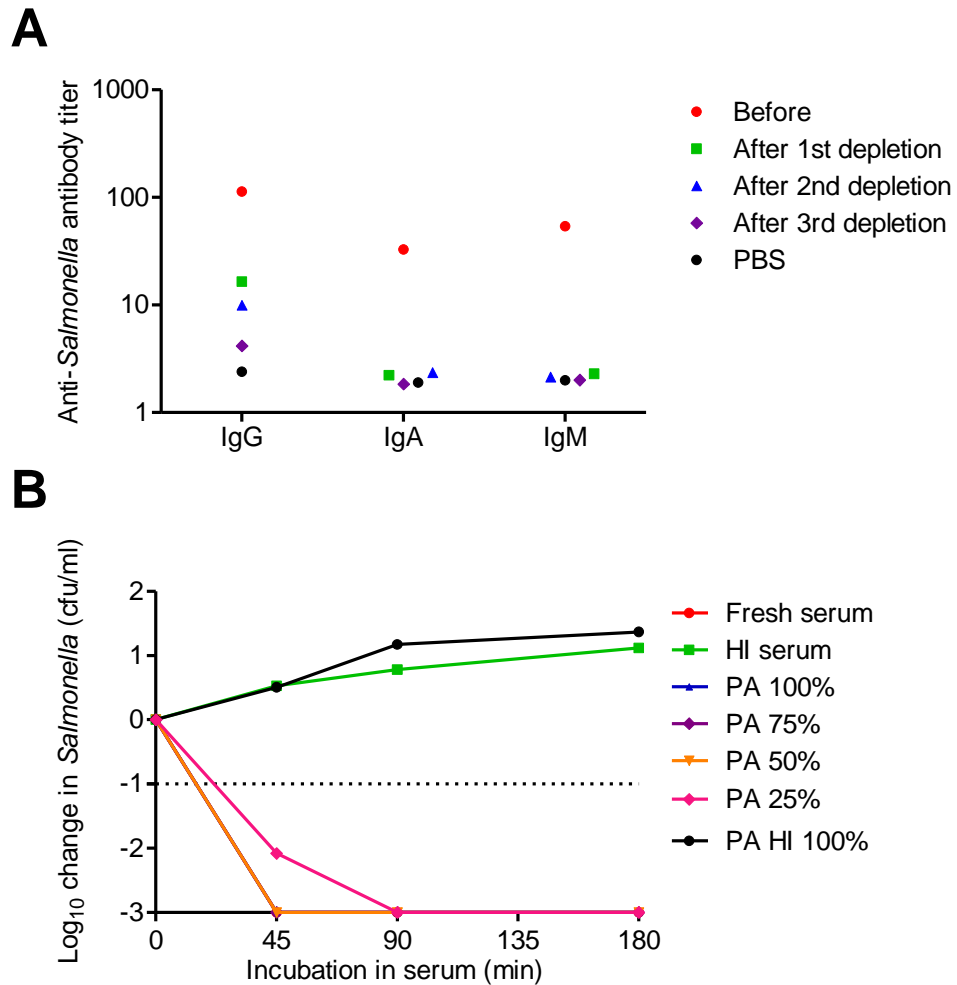


Figure 4.4 Antibody-depleted human serum contains no antibody to *Salmonella* but complement remains functional.

(A) Decreased anti-*S. Typhimurium* D23580 IgG, IgA and IgM deposition on *S. Typhimurium* D23580 in antibody-depleted serum after each round of absorptions compared to whole serum prior to pre-absorptions. (B) An SBA was performed with *S. Typhimurium* D23580 mutant *gale*⁻ and different percentages of pre-absorbed human serum (PA 100%, PA 75%, PA 50%, PA 25%) and compared to killing of bacteria with fresh non-pre-absorbed serum (Fresh serum). Pre-absorbed heat-inactivated serum (PA HI) and heat-inactivated non-pre-absorbed serum (HI serum) were negative controls. Initial concentration of *Salmonella* was 1×10^6 cfu/ml, with negative results showing a decrease in viable *Salmonellae* compared with the initial starting concentration. Data are from one experiment.

verify that complement within the antibody-depleted serum remains functional, a serum bactericidal assay using a *galE*- mutant of *S. Typhimurium* D23580 which has a truncated OAg and can be killed in the absence of antibody (50), was performed with pre-absorbed serum and whole serum. A 3.0 log₁₀ kill of *S. Typhimurium* D23580 *galE*- at 180 min was observed for whole non-pre-absorbed serum and for serum which had been through 3 rounds of antibody-depletion, even at percentages as low as 25% (Figure 4.4 B), confirming that complement remained functional in the pre-absorbed serum.

4.3.5 ANTIBODY-DEPLETED HUMAN SERUM IS A SUITABLE COMPLEMENT SOURCE FOR BLOOD CELL KILLING OF *S. TYPHIMURIUM* D23580

In contrast to BRS, all percentages of antibody-depleted serum tested did not induce *Salmonella* killing by peripheral washed blood cells in the absence of antibody (Figure 4.5 A). To determine whether killing of human antibody-depleted serum could be restored when supplemented with antibody, *Salmonellae* were opsonised with different percentages of antibody-depleted serum mixed with diluted heat-inactivated human serum and killing by washed blood cells measured (Figure 4.5 B). A minimum of 60% antibody-depleted serum was required to reliably induce a 1 log₁₀ kill of *Salmonella*, which is classed as 'normal' kill of *Salmonella* by healthy, adult serum. Opsonisation with 90% complement and 10% antibody induced a 1 log₁₀ kill after 180 min, but a 1:2 dilution of the antibody source (i.e. equivalent to 5% heat-inactivated serum) removed killing. When the complement source was reduced to 80% or 70%, a 1 log₁₀ kill was

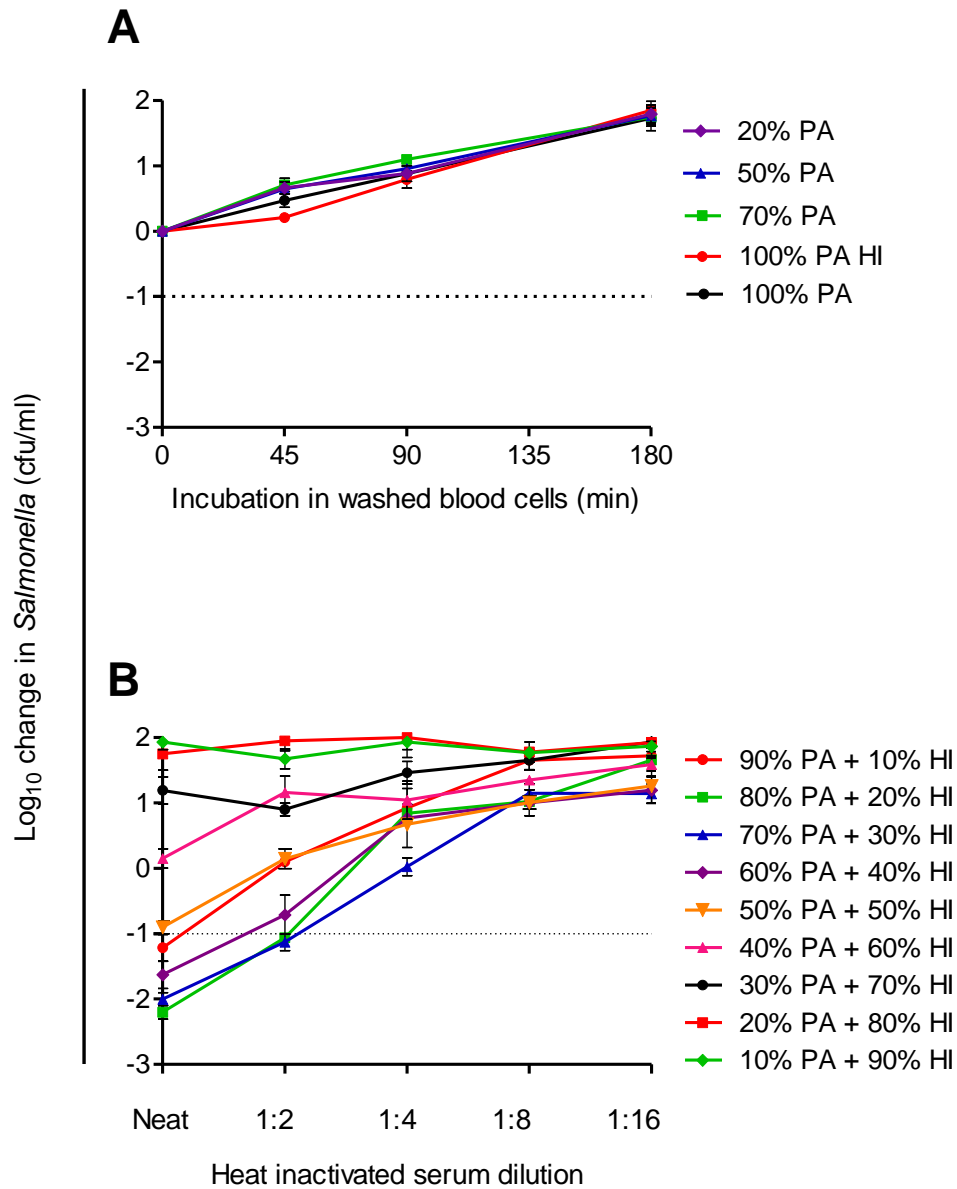


Figure 4.5 Antibody-depleted serum and antibody requirement for blood cell killing of *S. Typhimurium* D23580.

(A) *S. Typhimurium* D23580 was opsonised with 100%, 70%, 50%, 20% antibody-depleted serum (PA) or heat-inactivated antibody-depleted serum (100% PA HI) and killing by washed adult peripheral blood cells assessed at 45, 90 and 180 min. (B) Killing of *S. Typhimurium* D23580 opsonised with varying percentages of antibody-depleted serum (90%-10%) and supplemented with dilutions of heat-inactivated human serum as an antibody source (10%-90% neat, 1:2, 1:4, 1:8, 1:16). Neat corresponds to opsonisation with the maximum percentage of heat-inactivated serum possible, and dilutions correspond to 2 fold dilutions with PBS from this starting concentration. Log change in *Salmonella* after 180 min plotted against the antibody dilution, with negative results showing killing of *Salmonella* and positive results representing bacterial growth. Data represent means \pm SD of 3 experiments.

observed when antibody was diluted further to 1:2. 60% complement with 40% antibody induced over a 1.5 log₁₀ kill, but kill was decreased to 0.7 log₁₀ when diluted to 1:2. We suggest the use of 60% pre-absorbed serum, with 40% heat-inactivated serum as the antibody source for future blood cell killing assays.

4.3.6 COMPLEMENT AND ANTIBODY REQUIREMENT FOR SERUM BACTERICIDAL KILLING OF *S. TYPHIMURIUM* D23580 IS LOWER THAN THAT FOR BLOOD CELL KILLING

We had previously observed that human serum diluted to 20% can kill *Salmonella* via cell-free antibody-dependent complement-mediated killing (50)(Chapter 3, Figure 3.1). This suggests that the complement requirement for bactericidal killing is lower than that observed for blood cell killing, where fresh human serum, when diluted to 1:4, could not effect killing by peripheral blood cells (Figure 4.1 A, B). We confirmed that the limitation of serum bactericidal activity against *S. Typhimurium* D23580 for serum from the 2 donors used in this study was at a dilution of 1:4 (Figure 4.6 A and B).

To further identify differences in the antibody and complement requirement for the two different mechanisms of killing *Salmonella*, we compared the bactericidal activities of the same dilutions of antibody-depleted serum and heat-inactivated serum against *S. Typhimurium* D23580. As observed in the blood cell killing assay, pre-absorbed serum in the absence of antibody was not able to kill *Salmonella* (Figure 4.6 C). The minimal percentage of pre-absorbed serum required to induce a 1 log₁₀ bactericidal killing of

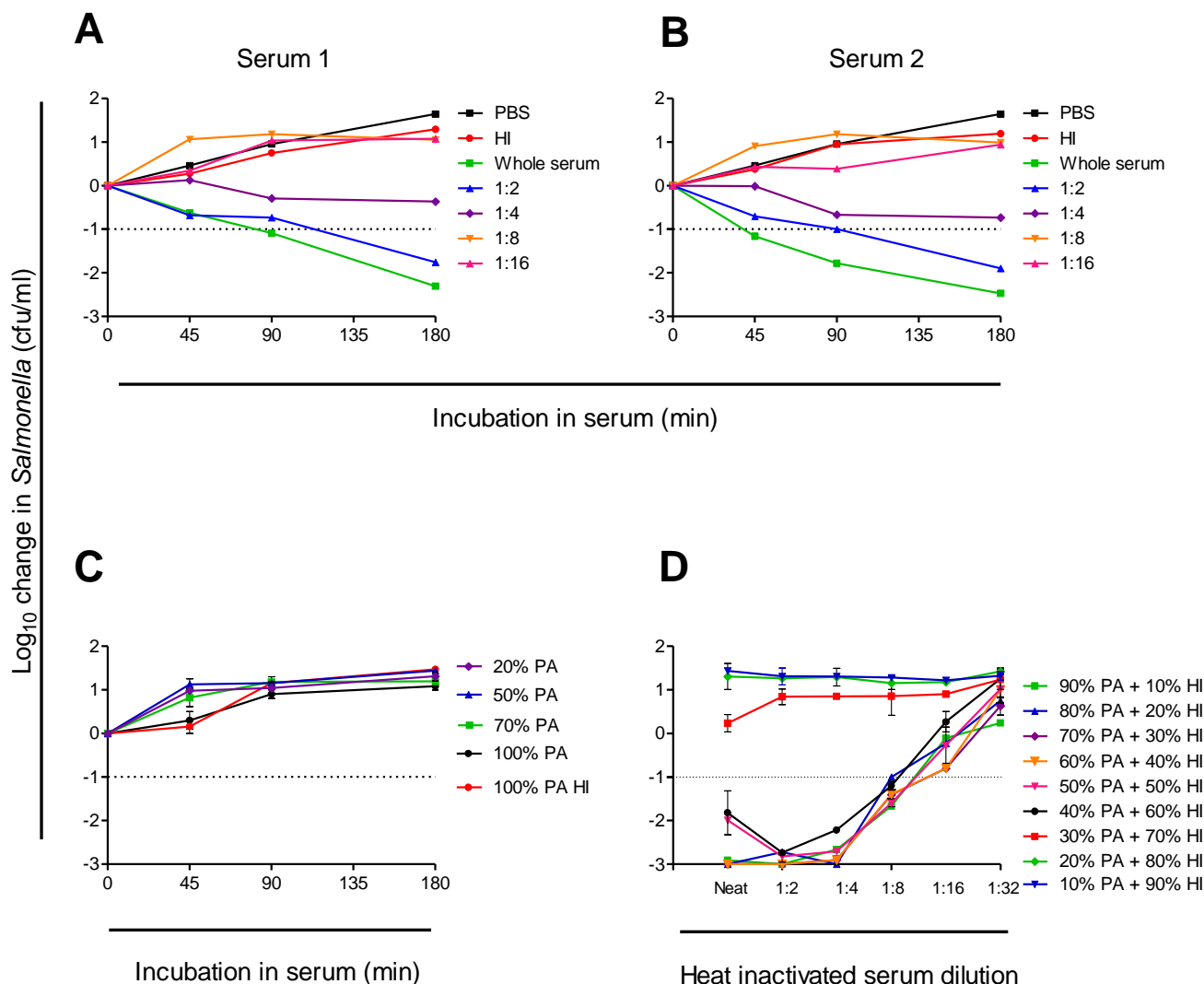


Figure 4.6 Requirement of antibody and complement for bactericidal killing of *S. Typhimurium*.

Serum bactericidal assays were performed using whole human serum from (A) donor 1 or (B) donor 2, or diluted with PBS (1:2, 1:4, 1:8, 1:16), or (C) varying percentages of antibody-depleted human serum (PA). Viable bacterial numbers were determined after 45, 90 and 180 min. (D) Serum bactericidal assays were also performed with varying percentages of antibody-depleted serum (90% PA-10% PA) and supplemented with dilutions of heat-inactivated human serum as an antibody source (10%-90% HI neat, 1:2, 1:4, 1:8, 1:16). Neat corresponds to opsonisation with the maximum percentage possible, and dilutions correspond to 2 fold dilutions with PBS from this starting concentration, with log change in *Salmonella* after 180 min plotted against the antibody dilution. Initial concentration of *Salmonella* was 1×10^6 cfu/ml, with negative results showing a decrease in viable *Salmonellae* compared with the initial starting concentration. A, B - each panel represents data using serum from one healthy adult. C, D - data represent means \pm SD of 3 experiments.

Salmonella in the presence of antibody was 40% (Figure 4.6 D). This is lower than 60% pre-absorbed serum required for killing of *Salmonella* by peripheral blood phagocytes. A 1 log₁₀ kill or greater was observed at antibody dilutions up to 1:8, with percentages of pre-absorbed serum ranging from 90% to 40%. At 70% and 60% complement, antibody could be diluted further and still induce a kill, with an antibody dilution of 1:16 producing a -0.8 log₁₀ *Salmonella* kill.

4.4 DISCUSSION

This work confirms an important requirement for antibody and complement in blood cell killing of *Salmonella*. We determined that endogenous complement in diluted human serum is insufficient to drive cell-mediated killing of *Salmonella*, and through the use of BRS and human antibody-depleted serum as exogenous complement sources, we evaluated the optimum percentages of complement and antibody required for peripheral blood cell killing of *Salmonella*.

Unlike for some diseases, such as meningococcal infections, where a proportion of the population lack antibody against the bacteria and can serve as complement donors (192), we have been unable to find healthy adult donors who lack naturally-acquired anti-*Salmonella* antibody, and we only have a limited amount of serum available from patients with primary antibody deficiencies. Therefore, we tested serum from 3-4 week old rabbits sourced from Pelfreez and AbD Serotec as potential complement sources. BRS is recommended by the WHO as a source of complement for measuring bactericidal

responses to meningococcal vaccines (193), and has been used also for assessing bactericidal activity to *S. Typhi* (196). The benefits of using commercial BRS are that it is readily available, requires no preparation and can be used in standardised assays and shared between different laboratories (192). Although no anti-*S. Typhimurium* D23580 antibodies were detected in either batch of BRS, it was toxic to *Salmonella* at high concentrations. At lower percentages of BRS, which were not toxic to *Salmonella*, killing could not be enhanced when supplemented with antibody, which rules out BRS as a complement source for our blood cell killing assays. It is likely that at low BRS percentages there is insufficient C3 deposition on the bacteria for phagocytosis. Lack of killing of *Salmonella* opsonised with high concentrations of BRS and incubated in RPMI, rather than blood, suggests that a component present in the blood causes the complement-dependent toxicity. It is possible that at high concentrations of BRS, there could be enough C3 deposition to trigger spontaneous uptake of the bacteria by blood phagocytes or that a little antibody remains in the washed blood cell suspensions, which together with deposition of sufficient quantities of BRS could stimulate phagocytosis.

The use of human complement avoids compatibility issues when using assay components from different species and has the benefit of reducing the use of animals, but more importantly, the use of human complement as well as human antibody and blood cells makes the assay conditions more closely related to those in vivo. A potential caveat of this pre-absorption method though is the batch to batch variation of this process and the time taken to deplete the serum of antibodies. One other concern is the loss of functional complement during the pre-absorption cycles, but we showed that

even 25% pre-absorbed serum was able to kill a *galE*- mutant of the parent strain D23580.

Human antibody-depleted serum was the most suitable complement source for use in our blood cell killing assay. A minimum of 60% pre-absorbed serum and 40% heat-inactivated serum at a maximum dilution of 1:2 (i.e. 60% complement and 20% antibody), is required to kill *Salmonella*. Human antibody-depleted serum was also a useful complement source for assessing cell-free, antibody-dependent complement-mediated killing of NTS. The percentage of complement and antibody required for killing *Salmonella* via this mechanism was much lower than that required for opsonic killing, with a minimum of 40% complement required and antibody dilutions of 1:8 and in some cases 1:16, able to effect bactericidal killing. The difference in requirement of antibody and complement using pre-absorbed serum and heat-inactivated serum mimics that seen with whole serum. Fresh whole serum at a maximum dilution of 1:2 could kill in the blood cell assay, but the same serum could be diluted to 1:4 for the SBA, suggesting a greater requirement for antibody and complement for killing by blood cells compared to serum bactericidal killing. This is surprising given that for phagocytosis, in relation to requirement for complement, only C3 deposition is needed to act as an opsonin but for bactericidal killing, formation of the MAC is required, so it might be expected that the complement requirement would be higher for the SBA, but for our two models the opposite is demonstrated. The increased requirement for antibody in blood cell killing compared to serum bactericidal killing could be explained by IgG being the only antibody class that can act as an opsonin whereas both IgG and IgM are

complement fixing and can trigger MAC formation, with IgM being highly effective at this.

We tested and defined the antibody and complement requirement for killing *S. Typhimurium* D23580, a representative invasive African NTS strain. However, the complement requirement could be different for other strains as it is known that different Malawian NTS isolates have varying susceptibility to opsonic antibody (see Chapter 8) or if a phagocytic cell line rather than fresh human peripheral blood cells are used. Furthermore, since the antibody titre can also affect the complement requirement, then higher antibody titres in some human serum, such as those post-vaccination, could alter the complement requirement. This work has potential implications for vaccine development, as to test the efficacy of an NTS vaccine we need an assay which can be used to assess the activity of vaccine-induced opsonic antibody. By identifying a suitable complement source and the percentage of complement and antibody required to induce killing by peripheral blood cells in our blood cell killing assay, we have identified an assay which can be used to measure both the specific role of natural and vaccine induced opsonic antibody in immunity to NTS.

4.5 CONCLUSIONS

Although commonly used as a source of exogenous complement, BRS was unsuitable for use in our *Salmonella* blood cell killing assay and antibody-depleted human serum was preferred as a complement source. Opsonisation with a minimum of 60% complement

and 40% antibody effectively killed *Salmonella*. In comparison with cell-mediated killing, the threshold of complement and antibody needed to induce bactericidal killing of NTS was lower, highlighting a difference in requirement for antibody and complement in killing of NTS by cellular and bactericidal mechanisms.

CHAPTER 5.

IMPAIRED CELL-MEDIATED IMMUNITY TO NTS IN HIV-INFECTED AFRICAN ADULTS

5.1 INTRODUCTION

Nontyphoidal *Salmonella* are a major cause of invasive bacterial disease in Africa affecting mainly young children (20;199) and HIV-infected adults (30). HIV-infected individuals with peripheral CD4 counts less than 200 cells/ μ l are susceptible to severe, recurrent invasive NTS disease (32) with resulting high mortality rates.

Salmonellae are facultative intracellular bacteria (84) and cellular mechanisms of immunity are important for controlling intracellular infection. The increased susceptibility to NTS infection of patients with immunodeficiencies in the IL-12/23-IFN γ axis (120;200) or with chronic granulomatous disease (111;112), who have mutations impairing oxidative burst activity, highlights this.

Antibodies also play a critical role in both bactericidal (50) and cellular (151) mechanisms of immunity to NTS. Sera from young African children lack antibody to NTS and consequently complement-mediated killing of the bacteria is diminished (50). Killing of *Salmonella* by the oxidative burst within phagocytes is also absent, as although these children do not have a deficiency in their oxidative burst mechanism, a lack of antibody means that phagocytosis, which is a necessary intermediate step for triggering the oxidative burst, is also reduced (151). Acquisition of antibody with age correlates

with a decline in NTS cases (50), highlighting the absolute requirement for complement and anti-*Salmonella* antibody in immunity to NTS.

In order to develop an effective vaccine against NTS for use in Africa, the targets of human antibodies against *Salmonella* need to be established. Studies in mice and rabbits have provided evidence for the protective efficacy of antibody against *Salmonella* outer membrane proteins (160), in particular OmpD (182). There is also evidence that antibodies against the O-antigen (OAg) of the lipopolysaccharide (LPS) molecule of *Salmonella* are protective (156-159;201;202). Passive transfer of monoclonal antibodies raised to smooth LPS, but not rough LPS, that lacks OAg, conferred significant protection in mice (201). In addition, immunising mice with *Salmonella* OAg conjugates elicited antibodies that protected mice against challenge (156-159;202).

However, there is a lack of evidence for a protective effect from serum LPS antibodies against NTS in humans. In fact, the opposite has been shown by MacLennan *et al* who observed dysregulated antibody-dependent complement-mediated immunity to NTS in some HIV-infected Malawian adults which have a higher titre of serum anti-LPS antibodies compared to HIV-uninfected Malawian adults (61). The study also showed that antibodies to the outer membrane proteins of *Salmonella* were bactericidal, and proposes that antibodies targeting the LPS of *S. Typhimurium* block bactericidal antibodies from binding to their targets on the outer membrane and /or directs complement away from the membrane, resulting in a lack of lysis of the bacteria (61). Whether high titres of serum anti-*S. Typhimurium* LPS antibodies inhibit cell-mediated

killing of NTS is unknown. Given that an OAg-conjugate vaccine is a potential vaccine candidate it is important to establish the role of anti-OAg antibodies in cell-mediated immunity in both healthy and HIV-infected African adults.

5.2 OBJECTIVES

The main objective of this study was to determine whether cell-mediated immune responses to NTS are impaired in HIV-infected African adults. We further identified if antibodies targeting the LPS molecule of *S. Typhimurium*, which have previously been shown to be inhibitory to antibody-dependent, complement-mediated immune mechanisms, also inhibit intracellular killing of *Salmonella* by blood phagocytes or whether they can be opsonic and induce clearance of *Salmonella* from the blood.

5.3 RESULTS

5.3.1 KILLING OF *S. TYPHIMURIUM* D23580 BY PERIPHERAL BLOOD CELLS IS IMPAIRED WHEN OPSONISED WITH SERUM FROM A SUBSET OF HIV-INFECTED AFRICAN ADULTS

We utilized a blood cell killing assay to determine any differences in killing of *S. Typhimurium* D23580 opsonised with HIV-uninfected and HIV-infected serum from Malawian adults by washed human peripheral blood cells. *Salmonella* Typhimurium D23580 was opsonised with serum from 3 different groups of Malawian adults (Table 5.1). The first group of sera was from five, HIV-uninfected healthy Malawian adults and

Group	HIV-uninfected	HIV-infected non-inhibitory	HIV-infected inhibitory
Number	5	5	5
Age (median and range)	26 (22-31)	38 (27-49)	27 (25-42)
Gender (male:female)	4:1	0:5	1:4
CD4 count cells/μl (median and range)	772 (329-1356)	110 (66-213)	105 (20-241)

Table 5.1 Clinical data for study participants.

are referred to as 'HIV-uninfected sera'. The second group was from 5 HIV-infected adults whose serum is able to effect a 1.0 - 3.0 log₁₀ kill of *S. Typhimurium* D23580 in the serum bactericidal assay, and these sera are referred to as 'HIV-infected non-inhibitory sera'. The third group was also from 5 HIV-infected Malawian adults, but these sera are referred to as 'HIV-infected (bactericidal) inhibitory sera' as they are unable to kill *S. Typhimurium* D23580 in the serum bactericidal assay (61). The HIV-infected adult participants were recruited from the antiretroviral clinic at QECH, Blantyre, Malawi and HIV-uninfected adults were hospital or laboratory staff. No participant had a history of *Salmonella* disease.

Washed blood cells from healthy non-HIV infected adults in the UK, were inoculated with opsonised *Salmonella* at a concentration of 1 x 10⁵ cfu/ml, at 37°C with viable *Salmonella* numbers determined at 45, 90 and 180 min. Washed human blood cell suspensions are used as endogenous complement and antibody have been removed from these, and therefore only the exogenous antibodies and complement present in the opsonising serum can bind to the *Salmonella*. Human blood cells are used rather than a phagocytic cell line to replicate human in vivo conditions as closely as possible.

The absolute requirement for both antibody and complement in killing *Salmonella* in this assay is shown by an absence of bacterial killing (2.0 log₁₀ growth) when *Salmonella* is opsonised with PBS or with antibody alone (heat-inactivated human serum) (Figure 5.1 A). This is in accordance with results of Gondwe *et al* who showed

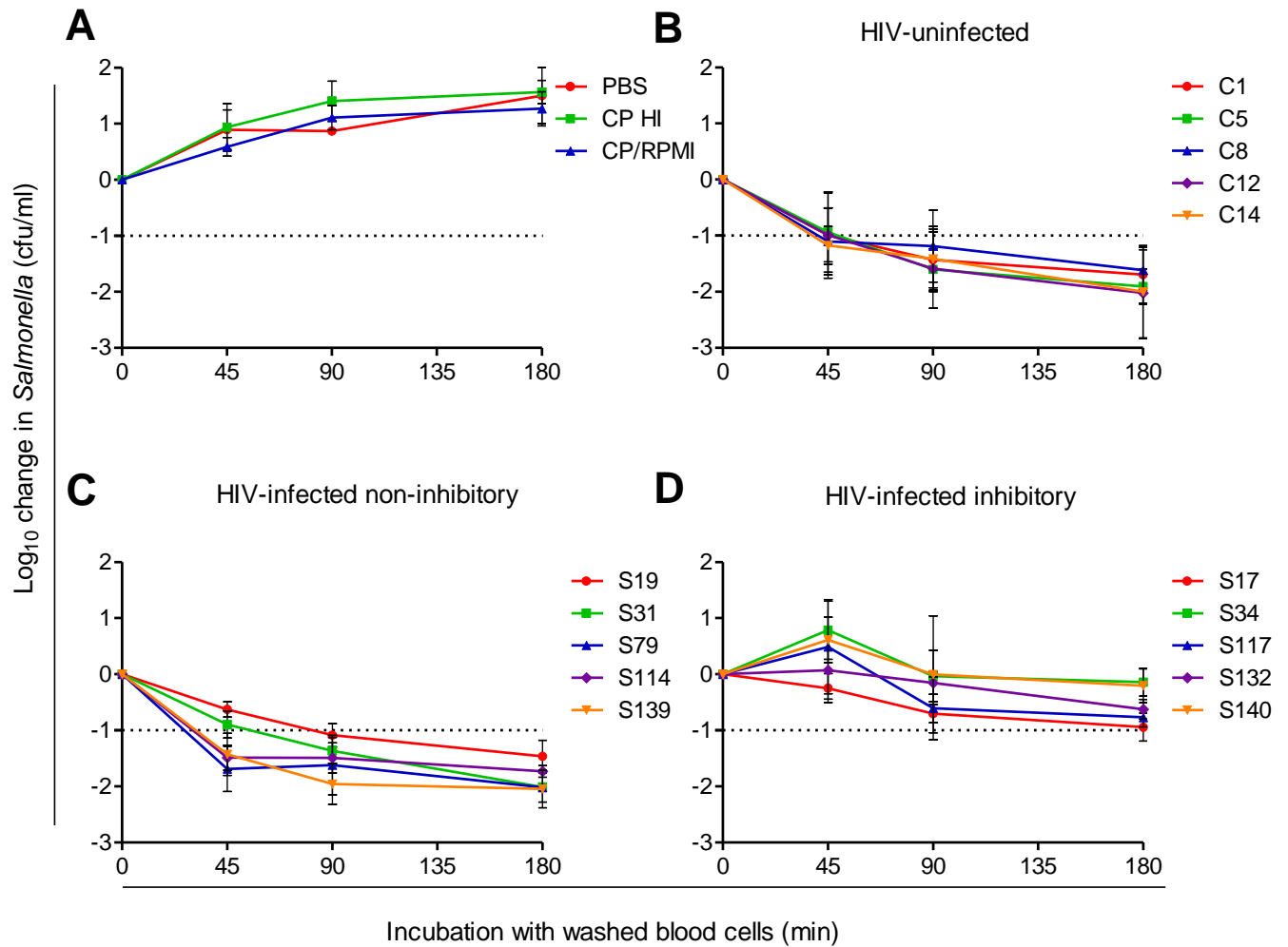


Figure 5.1 Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on killing of *Salmonellae* by adult washed peripheral blood cells.

In vitro killing of *Salmonella* opsonised with serum from HIV-uninfected Africans (B), non-inhibitory serum from HIV-infected African adults (C), and serum from HIV-infected African adults which is known to be inhibitory to bactericidal killing (D) at 45, 90 and 180 min. (A) shows PBS, heat-inactivated control pooled Malawian serum (CP HI), and control pooled serum as opsonins with *Salmonellae* incubated in RPMI rather than blood (CP + RPMI), showing the requirement for antibody and complement in opsonisation and that killing of *Salmonella* by this mechanism is cell dependent. Killing of *Salmonella* opsonised with HIV-infected bactericidal inhibitory serum is impaired compared to HIV-uninfected serum (45 min; un-paired t-test $p < 0.001$, 90 min; $p < 0.0001$, 180 min; $p < 0.0001$) Initial concentration of *Salmonella* is 1×10^5 cfu/ml, with negative results showing a decrease in viable *Salmonellae* compared with the initial starting concentration. C1-C14; Malawian HIV-uninfected sera, S19-S139; HIV-infected bactericidal non-inhibitory sera, S17-S140; HIV-infected bactericidal inhibitory sera. Data represent means \pm SD of 3 experiments.

that antibody and complement are required for killing of *Salmonella* by peripheral blood phagocytes from African children (151). An absence of killing when opsonised *Salmonella* is incubated in RPMI rather than washed blood cells confirms that killing of *Salmonella* in this assay is cell-mediated (Figure 5.1 A).

Salmonella opsonised with HIV-uninfected sera from African adults effected greater than a 1.5-2.0 log₁₀ kill after 180 min incubation with washed blood cells (Figure 5.1 B). A 1 log₁₀ kill at 180 min is designated 'normal kill' for serum from healthy HIV-uninfected adults in this assay. Opsonisation with serum from HIV-infected adults, which was not inhibitory in the bactericidal assays, effected a 2.0 log₁₀ kill after 180 min, similar to the kill effected with the HIV-uninfected sera (Figure 5.1 C). The difference in kill between the HIV-uninfected and HIV-infected non-inhibitory sera were not statistically significant at any time point (t45, un-paired t-test p=0.867; t90, p=0.767; t180 p=0.967). However, killing of *S. Typhimurium* D23580 opsonised with serum from HIV-infected adults, which was determined to be inhibitory in the serum bactericidal assays, was reduced compared to the other 2 groups (Figure 5.1 D). Bacterial growth was observed after 45 min, bacteriostasis at 90 min, and only 2 of the 5 sera effected a 1 log₁₀ kill of bacteria after 180 min (Figure 5.1 D). The impaired killing observed for the HIV-infected bactericidal inhibitory sera compared to the HIV-uninfected sera after 45 (un-paired t-test p<0.001), 90 (p<0.0001) and 180 min (p<0.0001) were all statistically significant.

5.3.2. PHAGOCYTOSIS OF *S. TYPHIMURIUM* D23580 OPSONISED WITH SERUM FROM SOME HIV-INFECTED AFRICAN ADULTS IS IMPAIRED

Killing of *Salmonella* in the previous blood cell assay is cell-mediated, suggesting that this mechanism of clearance from the blood is dependent on phagocytosis. Therefore, we next investigated if the opsonic potential of serum to stimulate phagocytosis of *S. Typhimurium* D23580 was impaired for sera from a subset of HIV-infected Malawian adults.

Heat-killed, FITC-labelled *Salmonella* Typhimurium D23580 were opsonised with PBS or sera from HIV-uninfected or HIV-infected adults, and the percentage phagocytosis by neutrophils and monocytes in washed peripheral human blood measured by flow cytometry. The mean phagocytic response by neutrophils and monocytes of *Salmonella* coated with PBS (no antibody or complement) was 3.65% and 2.15% respectively (Figure 5.2 A and B), emphasising the requirement of both antibody and complement in phagocytosis. Optimal phagocytosis of *Salmonella* was observed when *S. Typhimurium* D23580 were opsonised with sera from 5 HIV-uninfected adults (mean 80%) (Figure 5.2 A and B).

Stimulation of phagocytosis by *Salmonella* opsonised with HIV-infected non-inhibitory (mean 70%) serum was lower than that of non HIV-infected (mean 80%), but this difference was not statistically significant (un-paired t-test $p=0.16$ for neutrophils, $p=0.07$ for monocytes). Significantly less neutrophils (mean 63% versus mean 80%) and monocytes had phagocytosed *Salmonella* when opsonised with HIV-infected

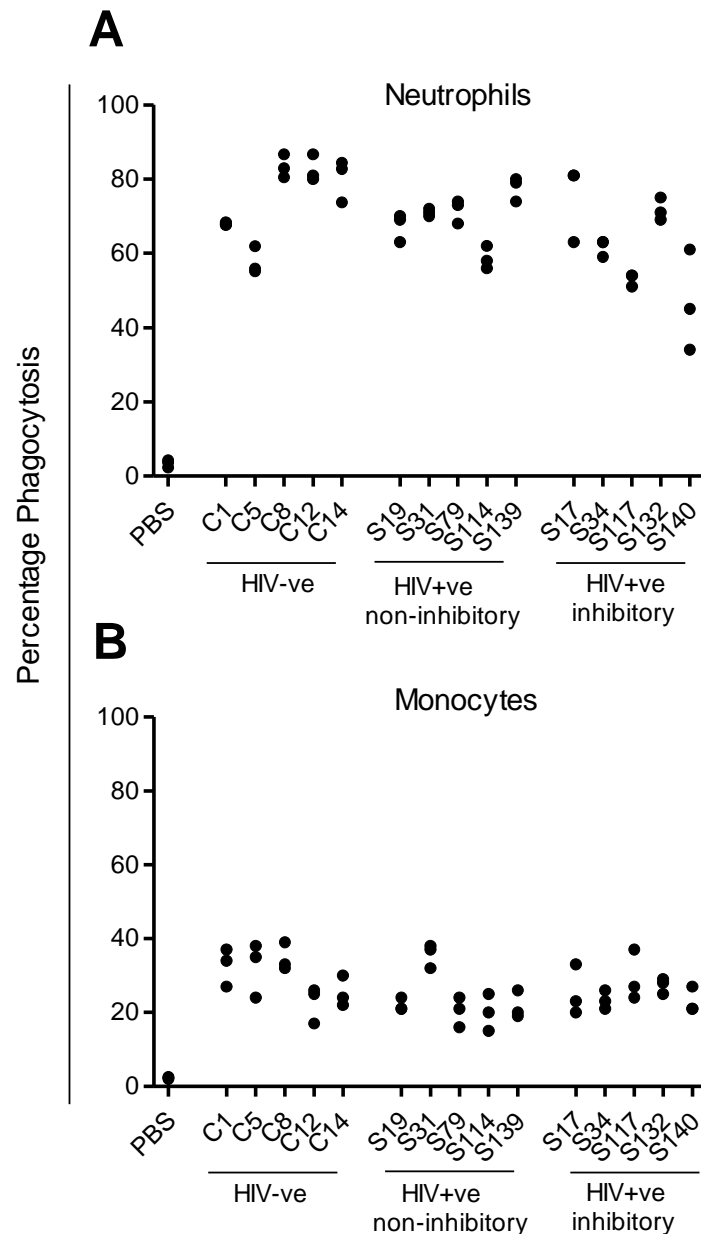


Figure 5.2 Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on phagocytosis of *Salmonellae* by adult washed neutrophils and monocytes.

(A) Percentage of phagocytosing washed neutrophils in response to FITC-labelled *Salmonellae* opsonised with PBS or serum from HIV-uninfected African adults (HIV-ve), was not significantly reduced (un-paired t-test $p=0.16$) compared to phagocytosis of *Salmonellae* opsonised with serum from HIV-infected non-inhibitory serum (HIV+ve non-inhibitory), but phagocytic response of neutrophils to stimulation with *Salmonellae* opsonised with serum from HIV-infected African adults (HIV+ve inhibitory) was significantly lower (un-paired t-test $p=0.008$). The response for monocytes using the same serum is shown in (B). Points in each group of 3 are from 3 separate experiments.

inhibitory serum (un-paired t-test $p=0.008$ for neutrophils and $p=0.02$ for monocytes) (Figure 5.2 A and B). The overall level of response by the monocytes (Figure 5.2 B) was lower than that of the neutrophils, and this was expected as blood had been taken from a healthy adult at rest so monocytes would not be activated. A lower response by monocytes was previously observed by Gondwe *et al* (151).

The result of this assay is in agreement with the observed impaired killing of *Salmonella* opsonised with HIV-infected bactericidal inhibitory serum. This suggests that the impaired clearance of *Salmonella* from the blood could be caused by a reduced uptake of *Salmonella* Typhimurium D23580 by neutrophils and monocytes.

5.3.3 OXIDATIVE BURST BY BLOOD PHAGOCYTES IS NOT REDUCED IN RESPONSE TO STIMULATION WITH *S. TYPHIMURIUM* OPSONISED WITH HIV-INFECTED OR HIV-UNINFECTED ADULT SERA

Once the bacteria have been phagocytosed, the oxidative burst is induced and this kills the intracellular bacteria. To assess whether the oxidative burst is also impaired in response to stimulus with *Salmonellae* opsonised with HIV-infected inhibitory sera, heat-killed *Salmonellae* were opsonised, and the oxidative burst response by washed human neutrophils and monocytes, measured by the conversion of dihydrorhodamine-1,2,3 to fluorescent rhodamine, was assessed by flow cytometry. Oxidative burst activity by both neutrophils and monocytes was absent when *S. Typhimurium* were opsonised with PBS (Figure 5.3 A and B), confirming the requirement for antibody and

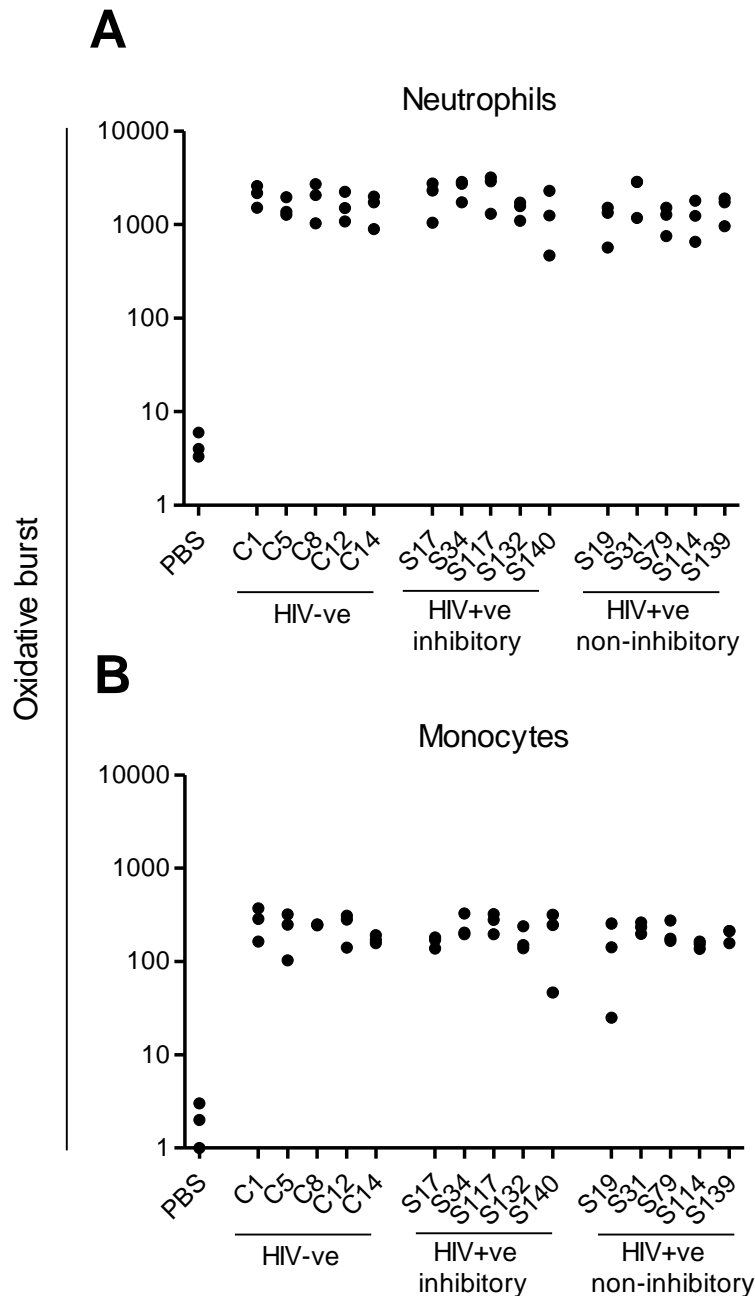


Figure 5.3 Effect of opsonisation with control and HIV-infected inhibitory and non-inhibitory sera on oxidative burst of *Salmonellae* by adult washed neutrophils and monocytes.

(A) Oxidative burst activity of washed neutrophils in response to *Salmonellae* opsonised with serum from healthy Africans (HIV-ve) and serum from HIV-infected African adults which is inhibitory to bactericidal killing (HIV+ve inhibitory), and serum from HIV-infected African adults which is not inhibitory to bactericidal killing (HIV +ve non-inhibitory) was not significantly different (neutrophils un-paired t-test $p=0.28$, monocytes un-paired t-test $p=0.36-0.43$). (B) The response for monocytes using the same sera. Points in each group of 3 are from 3 separate experiments.

complement for this mechanism of killing. The neutrophils produced a strong oxidative burst response (mean 1744 U for control, 1952 U for inhibitory, 1478 U for non-inhibitory) which was independent of the serum used to opsonise the bacteria (Figure 5.3 A). There was no statistically significant difference in oxidative burst activity between the HIV-uninfected serum and HIV-infected non-inhibitory (neutrophils, un-paired t-test $p=0.28$; monocytes $p=0.36$), or between the HIV-uninfected and HIV-infected inhibitory serum (neutrophils un-paired t-test $p=0.28$, monocytes $p=0.43$). The oxidative burst levels observed in the monocytes (Figure 5.3 B) were much lower than those observed for the neutrophils. Again, this was expected as the blood was taken from a healthy resting adult and so the monocytes would not be activated.

5.3.4 BACTERICIDAL KILLING OF *SALMONELLAE* DOES NOT OCCUR DURING THE OPSONISATION PERIOD

For the phagocytosis and oxidative burst assays, heat killed *Salmonellae* are used and consequently opsonisation with fresh serum cannot affect *Salmonellae* viability. For the blood cell killing assay, live bacteria are used and opsonisation takes place at room temperature for 20 min, prior to incubating with washed blood cells. Therefore, a possible explanation for the impaired blood cell killing, which was observed only with the HIV-infected bactericidal inhibitory serum, could be that bactericidal killing occurs during this opsonisation period, reducing the initial concentration of *Salmonella* at the start of the assay, but for the HIV-infected bactericidal inhibitory serum, this killing cannot take place (61) and so the concentration of bacteria at the start of the assay

would be higher for these samples. To verify if cell-free bactericidal killing occurs during this 20 min opsonisation period, *Salmonella* concentration was determined before and after the 20 min opsonisation period and the log change in *Salmonella* concentration calculated. There was a 0 log₁₀ change in *Salmonella* Typhimurium concentration at the end of the 20 min incubation for all of the sera tested (Figure 5.4) confirming that serum bactericidal killing of *S. Typhimurium* does not occur during the 20 min opsonisation period.

5.3.5 ALL SERA CAN DEPOSIT C3 ON *SALMONELLA*

Since both antibody and complement are required for optimal phagocytosis and killing of *S. Typhimurium* by peripheral blood cells, it was important to examine whether a lack of complement deposition was the reason for impaired killing observed when *Salmonellae* were opsonised with HIV-infected inhibitory serum. Using confocal microscopy, it had previously been verified that all 15 sera tested were able to deposit C3 on the surface of the bacteria (61), therefore excluding lack of complement deposition as an explanation for the impaired blood cell killing.

5.3.6 HIV-INFECTED INHIBITORY SERUM CAN KILL *S. TYPHIMURIUM* D23580 WHEN DILUTED WITH HIV-UNINFECTED SERUM

We wanted to verify if the inhibition of killing of *Salmonella* opsonised with HIV-infected inhibitory serum could be removed by mixing the inhibitory serum with HIV

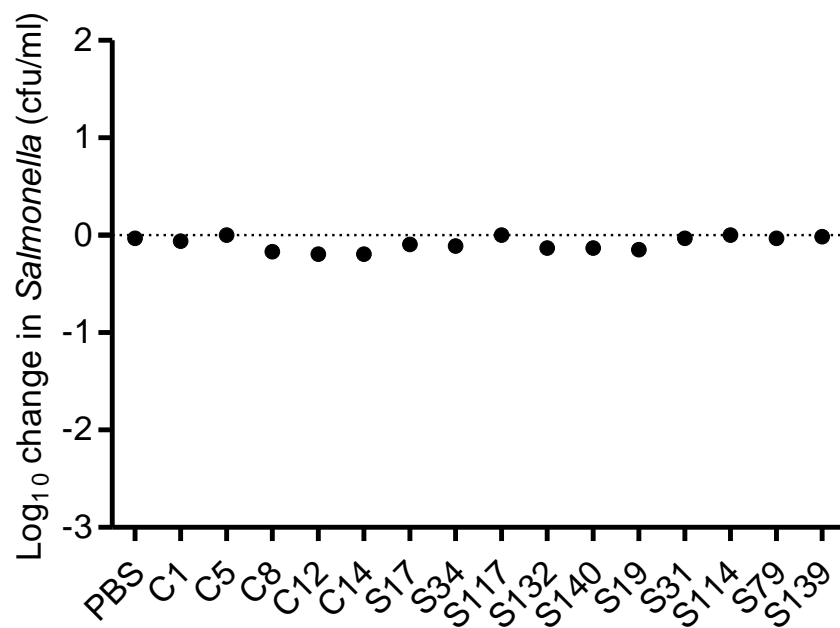


Figure 5.4 No bactericidal killing of *S. Typhimurium* D23580 occurs during the 20 min opsonisation period.

S. Typhimurium D23580 was opsonised with PBS (negative control) or 5 HIV uninfected, 5 HIV-infected inhibitory or 5 HIV-infected non-inhibitory sera and viable *Salmonella* counts taken at time 0 and after 20 min opsonisation period and log₁₀ change in *Salmonella* concentration plotted. Data are from one experiment.

un-infected serum which is able to effectively kill *Salmonella*. *S. Typhimurium* D23580 were opsonised with varying percentages of HIV-infected inhibitory and HIV-uninfected serum, and incubated with adult washed blood cell suspensions. Number of viable *Salmonella* after 45, 90 and 180 min was determined. Killing was restored in all of the HIV-infected inhibitory sera when mixed with the HIV-uninfected serum (Figure 5.5). For some sera, a 1 log₁₀ kill was only observed when *Salmonella* were opsonised with 40% HIV-infected inhibitory and 60% HIV-uninfected sera. For other sera, killing was restored when diluted to 80% inhibitory and 20% HIV-uninfected. This result is consistent with the presence of an inhibitor in the HIV-infected serum which affects the cell-dependent killing of *Salmonella*.

5.3.7 HIV-INFECTED INHIBITORY SERA HAVE HIGHER SERUM ANTI-LPS IgG AND IgA TITRES

We suspected that high anti-LPS antibody titres in the HIV-infected sera could be responsible for inhibiting *Salmonella* killing by washed blood cells. To test this we needed to determine the anti-LPS antibody titres of the serum. An ELISA was used to quantify IgG (Figure 5.6 A), IgA (Figure 5.6 B) and IgM (Figure 5.6 C) antibodies to *Salmonella* Typhimurium LPS in HIV-uninfected, HIV-infected inhibitory and non-inhibitory serum. No difference in anti-LPS IgG titres were seen for the HIV-infected non-inhibitory samples relative to the HIV-uninfected samples (un-paired t-test $p=0.656$), while the anti-LPS IgG titres for the HIV-infected inhibitory sera was significantly higher (un-paired t-test $p=0.0004$) (Figure 5.6 A). As for anti-LPS IgG, anti-LPS IgA titres were not statistically different for the HIV-infected non-inhibitory compared to HIV uninfected serum (un-paired t-test $p=0.875$), but the anti-LPS IgA

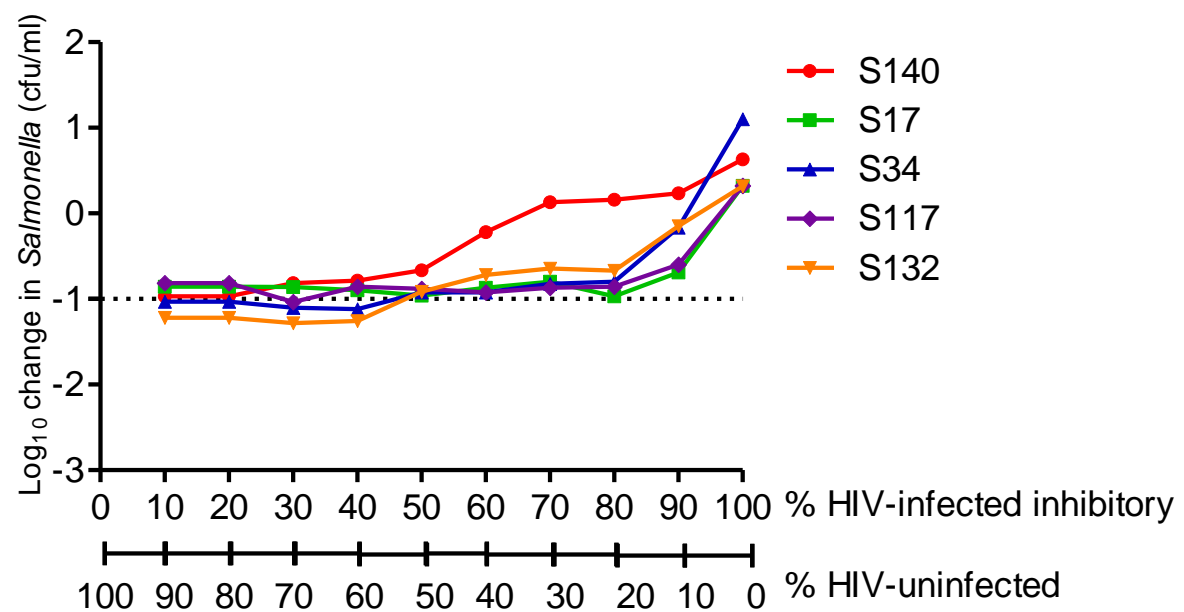


Figure 5.5 Inhibition of opsonic killing of *S. Typhimurium* by HIV-uninfected serum mixed with HIV-infected inhibitory serum.

S. Typhimurium D23580 was opsonised with different percentages of HIV-infected inhibitory serum and HIV-uninfected serum and incubated with washed blood cells and log change in *Salmonella* at 180 min shown. Each line represents data from one HIV-infected inhibitory serum.

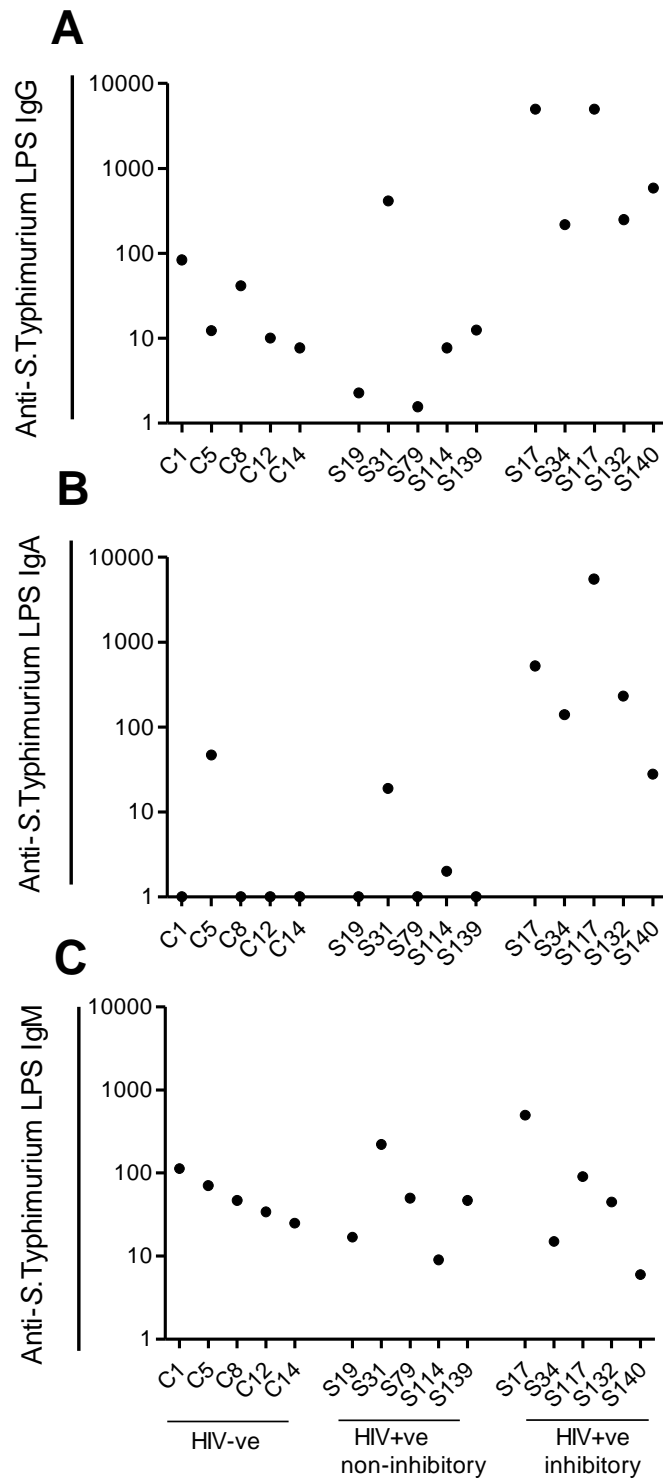


Figure 5.6 HIV-infected inhibitory sera have higher anti-*S. Typhimurium* LPS IgG and IgA titres.

Anti-*S. Typhimurium* IgG (A), IgA (B) and IgM (C) titres of 5 sera from HIV uninfected Malawian adults (HIV-ve), 5 HIV-infected non-inhibitory (HIV+ve non-inhibitory) and 5 HIV-infected inhibitory adult sera (HIV+ve inhibitory) were quantified using *S. Typhimurium* LPS coated ELISA plates. Anti-*Salmonella* LPS IgG and IgA titres were significantly higher in the HIV-infected inhibitory sera compared to the HIV-uninfected sera (un-paired t-test, $p=0.0004$, $p=0.0182$). Data are from one experiment.

titres for the HIV-infected inhibitory serum were significantly increased (un-paired t-test $p=0.018$) (Figure 5.6 B). There was no difference in anti-LPS IgM titres between the HIV-uninfected and HIV-infected non-inhibitory serum (un-paired t-test $p=0.655$) or HIV-infected inhibitory serum ($p=0.861$) (Figure 5.6 C).

5.3.8 *S. TYPHIMURIUM* MUTANT *gale*- OPSONISED WITH SERUM FROM MALAWIAN ADULTS IS KILLED BY PERIPHERAL BLOOD CELLS

We suspected that high titres of anti-*S. Typhimurium* LPS antibodies which are found in a subset of HIV-infected adults were inhibiting the killing of *Salmonella Typhimurium*. Using a mutant of *Salmonella Typhimurium* D23580, which has a mutation in the *gale* gene, leading to a truncation in the OAg chain of the LPS molecule, then the effect of antibodies targeting the OAg could be determined. In theory, if high titres of antibody targeting the OAg of *S. Typhimurium* block the killing of bacteria, then all sera should be able to opsonise and kill the *gale*- mutant.

The *gale*- mutant of *S. Typhimurium* D23580 was opsonised with PBS (as a negative control), HIV-uninfected, HIV-infected non-inhibitory and HIV-infected inhibitory serum prior to incubating with washed blood cells. The number of viable bacteria were determined after 45, 90 and 180 min incubation in the blood. *S. Typhimurium gale*- opsonised with PBS was not killed by washed blood cells (Figure 5.7 A). There was a 1000 fold decrease ($3.0 \log_{10}$ kill) in bacteria after 180 min opsonised with each serum (Figure 5.7 A, B, C). It is unclear why opsonisation of *S. Typhimurium gale*- with one of

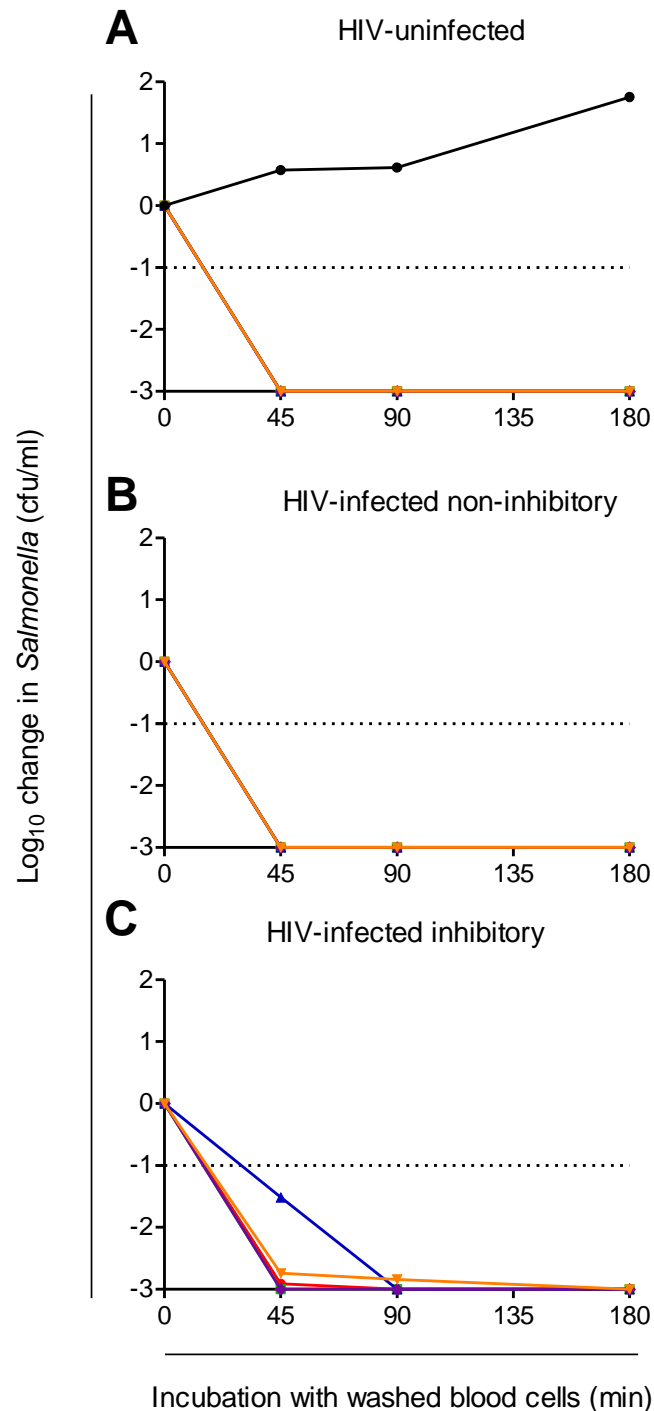


Figure 5.7 Effect of opsonisation with HIV-uninfected and HIV-infected inhibitory and non-inhibitory sera on killing of *Salmonellae* Typhimurium D23580 *galE*- mutant which has a truncated OAg chain by adult washed peripheral blood cells.

In vitro killing of *S. Typhimurium* D23580 *galE*- mutant opsonised with (A) PBS (black line) and serum from HIV-uninfected Africans, (B) non-inhibitory serum from HIV-infected African adults, and (C) serum from HIV-infected African adults which is known to be inhibitory to bactericidal killing, at 45, 90 and 180 min. Initial concentration of *Salmonellae* is 1×10^5 cfu/ml, with negative results showing a decrease in viable *Salmonellae* compared with the initial starting concentration. Data are from one experiment.

the HIV-infected inhibitory sera (S117) is reduced at t45 and t90 min when compared to the other sera, although this serum has the highest anti-LPS antibody titre. This result suggests that all the sera have an inherent ability to opsonise *S. Typhimurium* D23580 and that the presence of excess anti-LPS antibodies in serum from some HIV-infected adults, which target the OAg of *Salmonella* inhibit killing of the wild type strain.

5.3.9 REMOVAL OF LPS ANTIBODIES RESTORES KILLING OF *SALMONELLA* OPSONISED WITH HIV-INFECTED INHIBITORY SERUM

In order to further test whether antibodies to the LPS of *Salmonella* Typhimurium were inhibiting killing, we examined the effect of removing anti-LPS antibodies from HIV-uninfected (Figure 5.8 A) and two HIV-infected inhibitory serum (Figure 5.8 B, C) on killing by peripheral blood cells. We found that purified *S. Typhimurium* LPS affects blood cell viability, most likely through the toxic effects of lipid A. Therefore, we could not use LPS added to the serum to absorb antibodies prior to opsonisation. Removal of anti-LPS antibodies was achieved by incubating serum in an ELISA plate coated with purified *Salmonella* Typhimurium LPS. Serum which had undergone 1, 6 or 12 incubations with LPS were tested in an ELISA to confirm that antibody had been removed, and the titre of anti-LPS antibody decreased in a step-wise fashion with very little antibody against LPS remaining after 12 incubations. The pre-absorbed sera were used to opsonise *S. Typhimurium* D23580 before incubating with washed blood cells. Viable bacteria were counted after 45, 90 and 180 min incubation with washed blood cells and log change in bacteria determined.

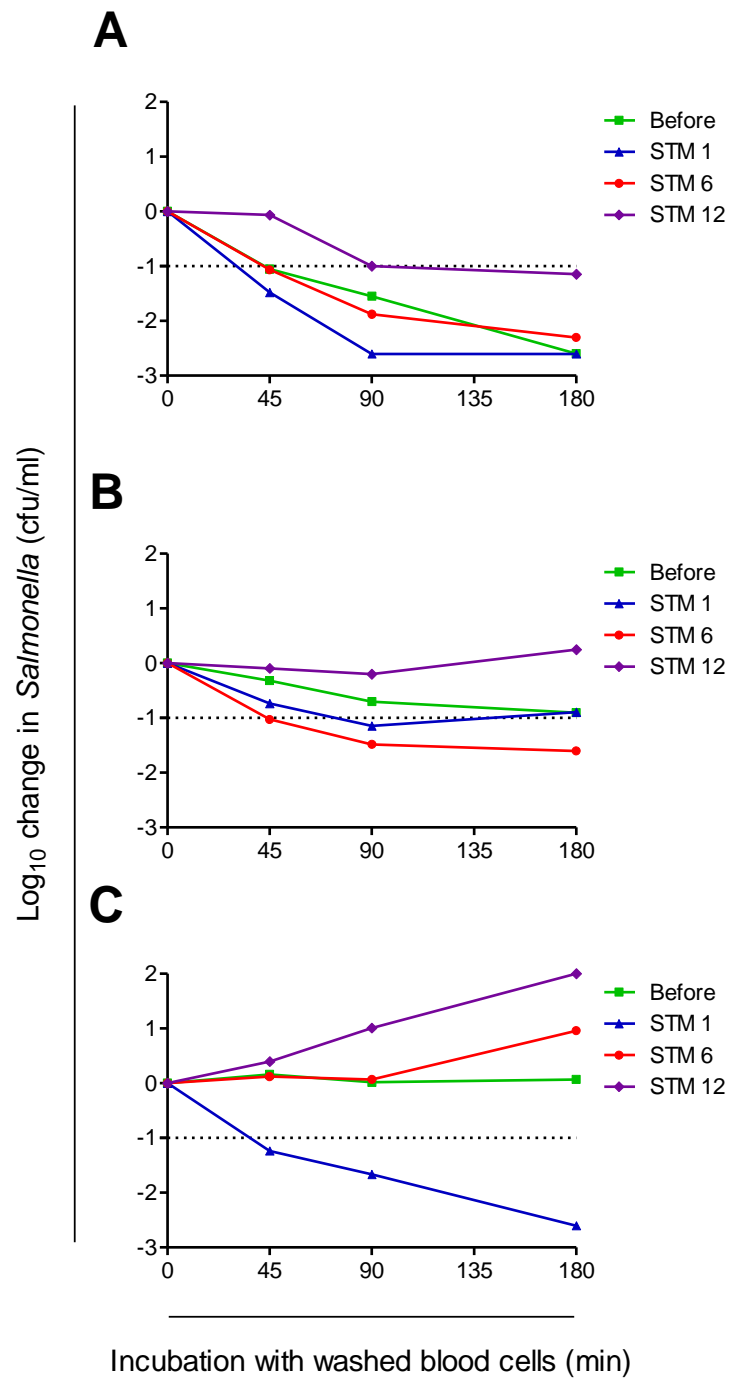


Figure 5.8 Effect of opsonising *S. Typhimurium* D23580 with HIV-uninfected or HIV-infected sera pre-absorbed with *S. Typhimurium* LPS.

(A) Blood cell killing of *S. Typhimurium* opsonised with sera from (A) HIV-uninfected or (B, C) 2 HIV-infected inhibitory sera which had undergone 1 (STM 1), 6 (STM 6) or 12 (STM 12) pre-absorption cycles with *S. Typhimurium* LPS to remove anti-LPS antibodies. Whole serum (before) which has undergone no pre-absorption cycles is included for comparison. Each panel represents data using serum from one African adult.

Killing of *Salmonella* opsonised with fresh HIV-uninfected sera (Figure 5.8 A) was enhanced after one pre-absorption cycle, compared to opsonisation with non-pre-absorbed serum. But opsonisation with HIV-uninfected serum after 6 or 12 pre-absorption cycles did not enhance the killing, with a reduced kill for *Salmonella* opsonised with serum after 12 pre-absorption cycles compared to non-pre-absorbed serum. Removal of anti-LPS antibodies from two HIV-infected inhibitory sera tested (Figure 5.8 B and C) also enhanced the killing, but removal of more antibody after 6 (Figure 5.8 C) or 12 (Figure 5.8 B) cycles reduced the killing.

This observation is consistent with the hypothesis that high titres of anti-LPS antibodies are responsible for the impaired killing observed in the phagocytosis and blood cell killing assays, since when the titre of anti-LPS antibody decreases, killing of *Salmonella* opsonised with these sera is enhanced. However, the results also indicate that removal of too much anti-LPS antibody decreases killing, suggesting that anti-LPS antibodies have a role in cell-mediated immunity and that it is the concentration of anti-LPS antibody that impairs killing.

5.3.10 REMOVAL OF ANTIBODIES TO LPS FROM DIFFERENT SEROVARS OR COMPONENTS OF LPS *S. TYPHIMURIUM* DOES NOT RESTORE KILLING OF *S. TYPHIMURIUM* D23580 BY WASHED BLOOD CELLS

It was important to confirm that the target antigen of the inhibitory antibodies is the OAg of LPS, and that the enhanced killing of *Salmonella* opsonised with anti-LPS antibody depleted serum was due to the removal of antibodies to the OAg chain rather

than antibodies targeting the core or lipid A of LPS. To test this, we repeated the pre-absorption cycles using purified lipid A and Rb, Rc, Rd and Re rough mutants of *Salmonella* as antigens. These *Salmonella* rough mutants have varying truncations in the core region of LPS. Killing of *Salmonella* Typhimurium D23580 opsonised with HIV-infected inhibitory serum from either of two adults with antibodies to lipid A or the core oligosaccharides removed was still impaired (Figure 5.9 A, B) and killing could not be restored after 6 pre-absorption cycles. This indicates that the inhibitory antibodies specifically target the OAg of *Salmonella* Typhimurium.

Blood-cell mediated killing of *Salmonella* opsonised with HIV-infected inhibitory serum which had undergone 6 pre-absorption cycles using purified LPS from *Salmonella* Enteritidis or *Salmonella* Minnesota did not restore killing either (Figure 5.9 C, D). *S. Enteritidis* belongs to group D *Salmonella* and *S. Minnesota*, group L, and the O-antigens for these three serovars are known to be non-cross-reactive suggesting that it is antibody targeting the OAg of *S. Typhimurium* (i.e. anti-O:4,5 antibodies) which inhibits the killing of *S. Typhimurium* by serum from some HIV-infected adults.

5.3.11 HIGH CONCENTRATIONS OF PURIFIED O-ANTIGEN ANTIBODIES INHIBIT CELL-MEDIATED KILLING OF *S. TYPHIMURIUM* D23580

We obtained affinity purified OAg *S. Typhimurium* antibodies from 3 sera from each group of subjects (see Chapter 6). The purified anti-OAg antibodies allow us to further

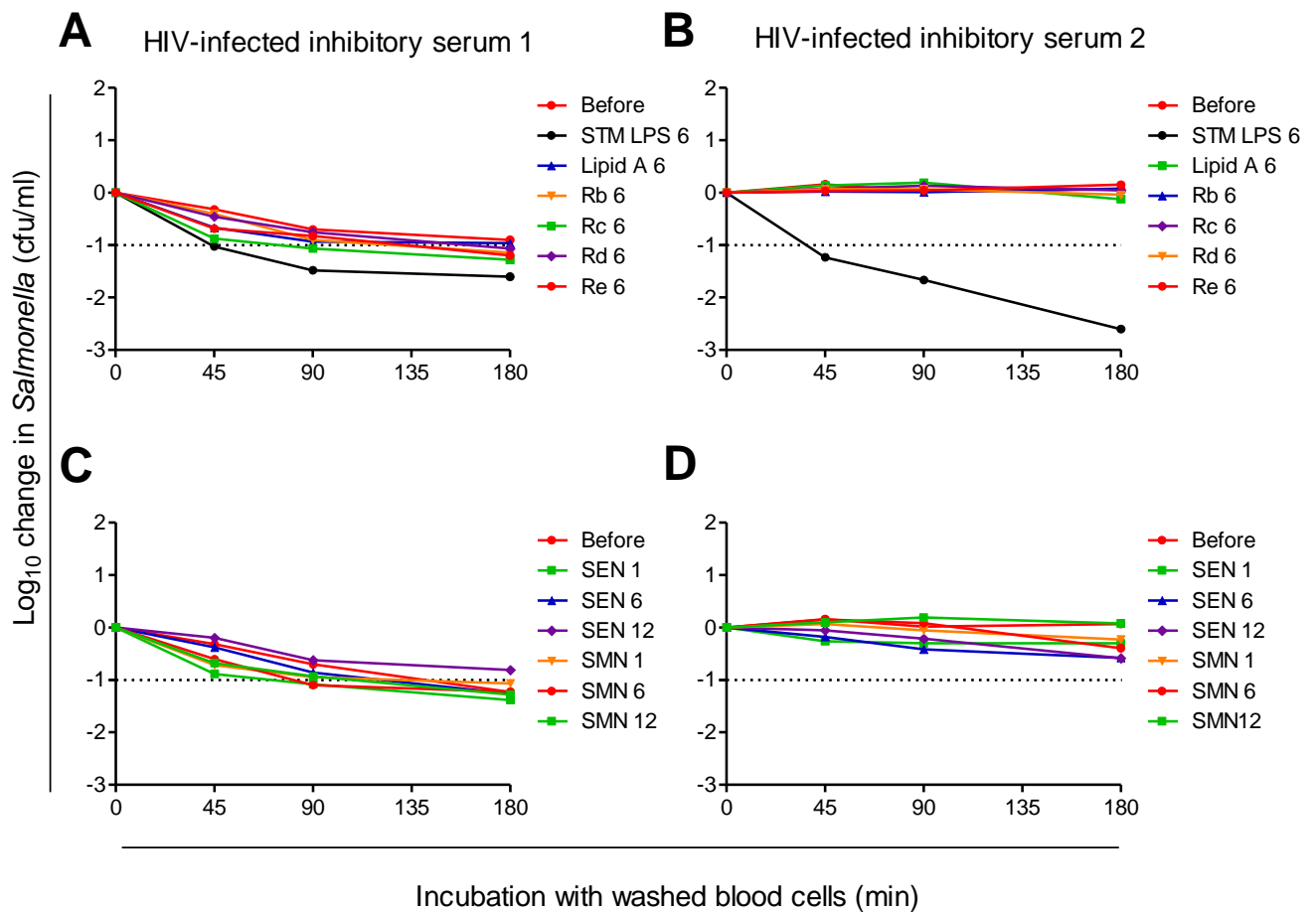


Figure 5.9 Effect of pre-absorbing HIV-infected sera with different components of *Salmonella* LPS and LPS from *S. Enteritidis* and *S. Minnesota* on blood cell killing of *S. Typhimurium* D23580.

(A, B) Two HIV-infected inhibitory sera underwent 6 pre-absorption cycles with *Salmonella* lipid A and LPS from Rb, Rc, Rd, Re – *Salmonella* mutants with truncations in the core oligosaccharide, prior to opsonising *S. Typhimurium* D23580 and incubating with washed blood cells. Serum which had not been pre-absorbed (Before) was used as a control and 6 pre-absorption cycles with *Salmonella* Typhimurium LPS (STM LPS 6) shown for comparison. (C and D) Effect of 1, 6 or 12 *S. Enteritidis* LPS (SEN) and *S. Minnesota* LPS (SMN) pre-absorption cycles on 2 HIV-infected inhibitory sera prior to opsonisation of *S. Typhimurium* D23580. Initial *Salmonella* concentration 1×10^5 cfu/ml with decrease *Salmonellae* showing killing of bacteria. Data are representative of one experiment with sera from 2 HIV-infected Malawian adults.

characterise the role of OAg antibodies in cell-mediated immunity to NTS. *S. Typhimurium* D23580 opsonised with 50% fresh HIV-uninfected serum and 50% PBS undergoes a 2.0 log₁₀ kill by washed peripheral blood cells, providing a model into which we can test the effect of anti-OAg antibodies. In theory, if anti-OAg antibodies are responsible for inhibiting cell-mediated killing of NTS then killing of *Salmonella* opsonised with 50% HIV-uninfected fresh serum and 50% OAg antibody will be reduced. Opsonisation of *S. Typhimurium* D23580 with 50% serum and 50% purified LPS antibodies added at the titre in the source serum, or diluted with PBS, from 3 HIV-uninfected serum did not affect the killing of *Salmonella* (Figure 5.10 A, B, C). The same level of kill was effected whether bacteria were opsonised with 50% fresh serum and 50% PBS, or 50% serum and 50% purified OAg antibody from, and adjusted to the concentration found in HIV-infected non-inhibitory serum (Figure 5.10 D, E, F). However, inhibition of *Salmonella* killing by blood cells was observed when *Salmonella* were opsonised with 50% serum and 50% OAg antibodies purified from 3 HIV-infected inhibitory sera (Figure 5.10 G, H, I). This inhibition did not occur with sufficient dilution of the purified OAg antibody. Figure 5.10 G, shows that the inhibitory effect of the OAg antibodies was not present when using a 1:64 dilution of these antibodies. For the 2 other HIV-infected inhibitory serum, the purified OAg antibodies needed to be diluted to 1:8 (Figure 5.10 H) and 1:16 (Figure 5.10 I) before killing of *Salmonella* was similar to that for bacteria opsonised with fresh serum and PBS rather than fresh serum and OAg antibodies. This established that inhibition of killing by OAg antibodies is concentration-dependent.

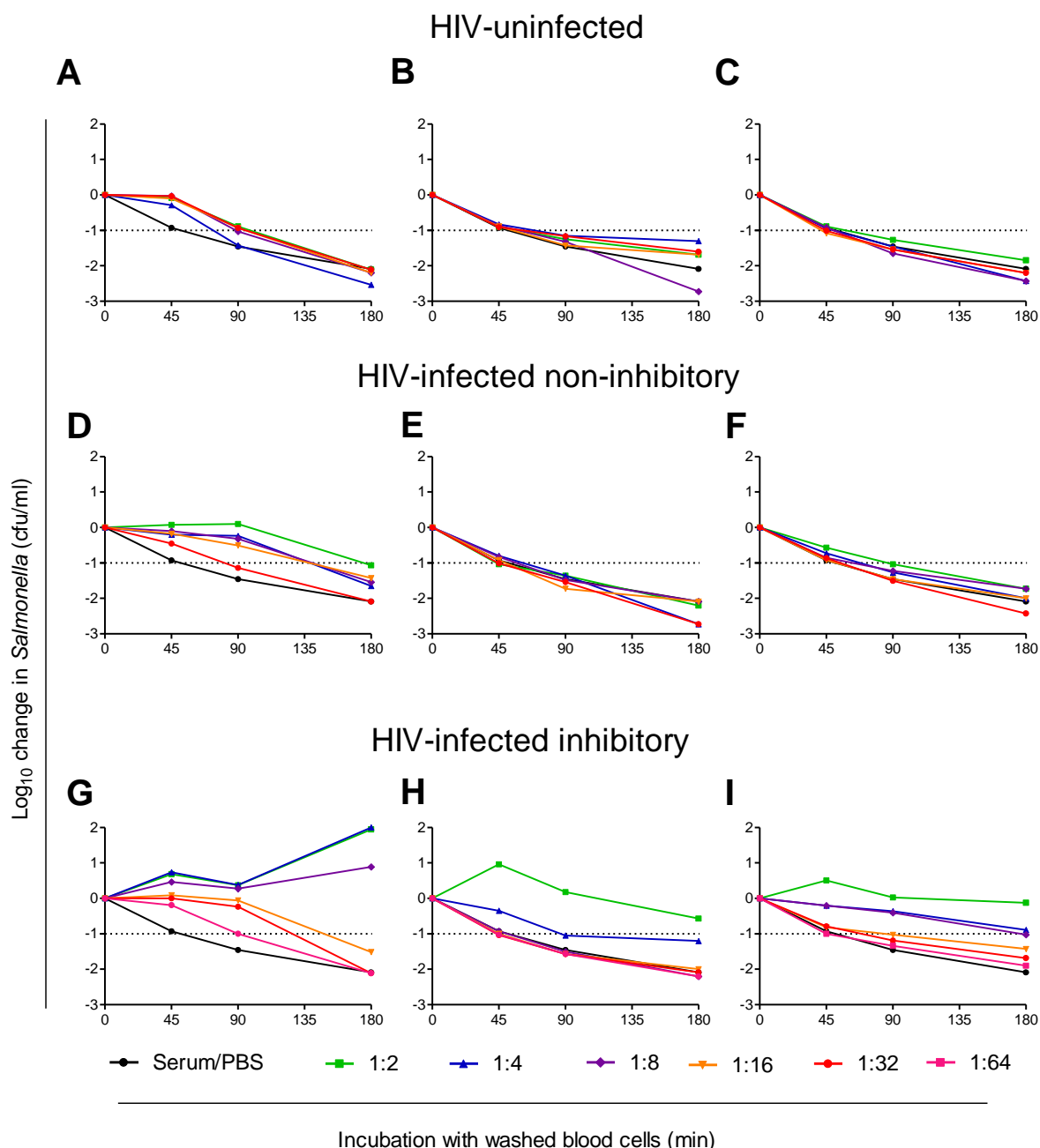


Figure 5.10 Effect of anti-OAg antibodies on killing of *S. Typhimurium* D23580 by washed blood cells.

S. Typhimurium D23580 was opsonised with 50% fresh HIV-uninfected serum and 50% PBS (Serum/PBS) or 50% fresh HIV-uninfected serum and 50% purified anti-OAg antibodies from 3 HIV-uninfected African adult sera (A,B,C), 3 HIV-infected non-inhibitory sera (D,E,F) or 3 HIV-infected non-inhibitory serum (G, H, I). Purified anti-OAg antibodies were added at the titre in each of the sera they are purified from (e.g. 1:2; 50% fresh HIV-uninfected serum, 50% purified OAg antibody) and then diluted 2 fold with PBS (1:4 (50% fresh HIV-uninfected serum, 25% purified OAg antibody, 25% PBS), 1:8, 1:16, 1:31, 1:64). Each panel represents OAg antibodies purified from the sera of one African adult.

Purified OAg antibodies from these HIV-infected inhibitory sera were at a higher concentration than those from HIV-uninfected or non-inhibitory serum which suggested it is the titre, and not the source, of anti-OAg antibodies which inhibits blood cell killing of *Salmonella*. To confirm this, OAg antibodies purified from HIV-uninfected serum were concentrated to the titre found in the HIV-infected inhibitory serum. *Salmonellae* were opsonised with 50% fresh serum and 50% purified OAg antibodies, and killing by blood cells determined. When *Salmonellae* were opsonised with these antibodies then the killing was impaired with a 2.0 log₁₀ growth observed (Figure 5.11 A). Normal killing occurred when these antibodies were diluted with PBS to 1:8 of the starting concentration. To further test this, *Salmonella* were opsonised with 50% fresh serum and 50% OAg antibody purified from a HIV-infected inhibitory serum, but diluted to the titre of OAg antibody found in HIV-uninfected serum. This time these antibodies, although from the HIV-infected inhibitory serum, were able to kill *Salmonella*, even at the first dilution used as the titre was already adjusted to that of the HIV-uninfected serum (Figure 5.11 B). This confirmed that it is the high titre of antibody found in some HIV-infected serum which reduces killing of *Salmonella* by blood cells and not a difference in antibodies between HIV-infected and HIV un-infected serum.

5.3.12 PURIFIED ANTI-SALMONELLA TYPHIMURIUM O-ANTIGEN ANTIBODIES HAVE AN OPSONIC FUNCTION

Using human serum which had anti-*Salmonella* antibodies removed (see Chapter 4) as a complement source, we were able to identify whether anti-S. Typhimurium OAg

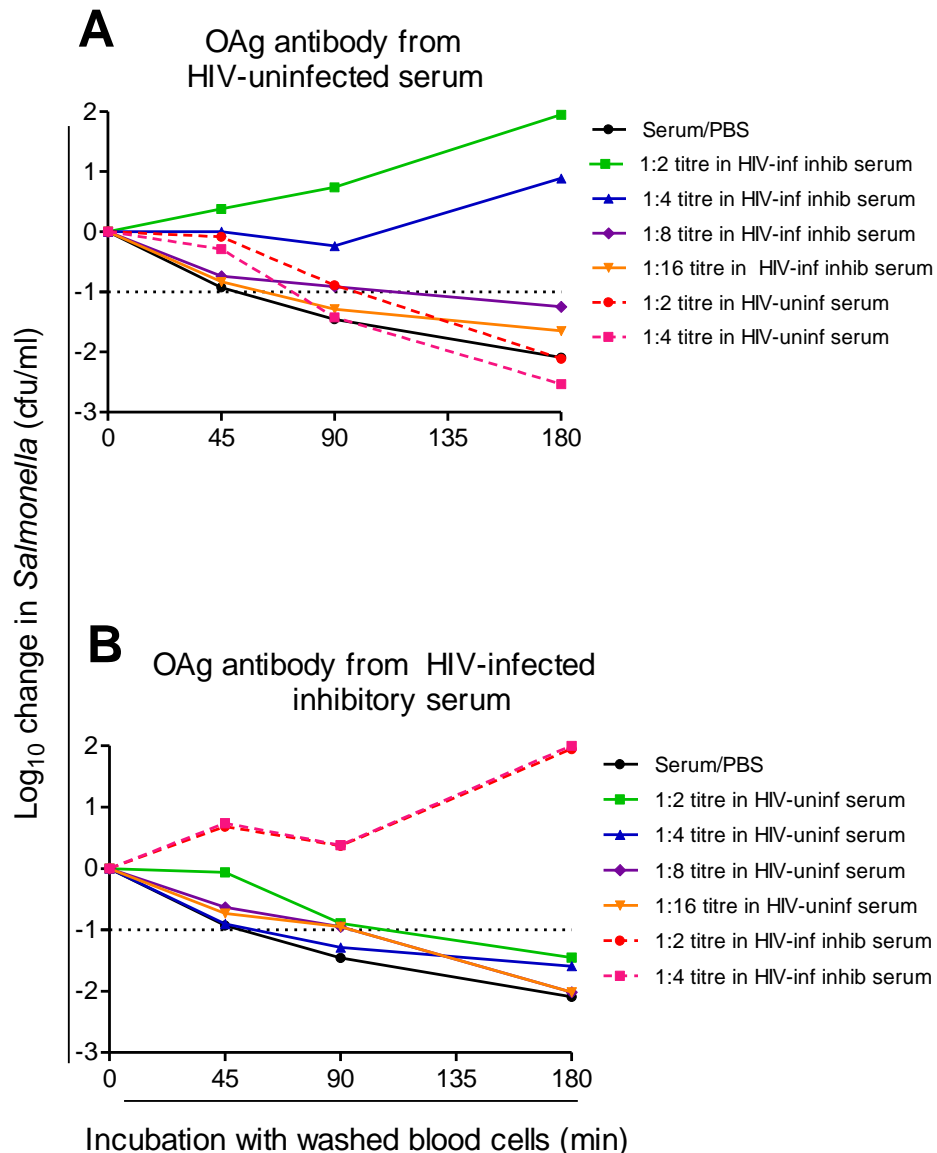


Figure 5.11 Effect of anti-OAg antibodies on killing of *S. Typhimurium* D23580 by washed blood cells.

(A) *S. Typhimurium* D23580 was opsonised with 50% fresh HIV-uninfected serum and 50% PBS (Serum/PBS), or 50% fresh HIV-uninfected serum and 50% purified anti-OAg antibodies from HIV-uninfected African adult sera but concentrated to the titre found in HIV-infected inhibitory serum (1:2 titre in HIV-inf inhib serum), and then diluted 2 fold with PBS (1:4 titre in HIV-inf inhib serum, 1:8, 1:16). Effect of killing of anti-OAg antibodies purified from the same HIV-uninfected serum and concentrated to the titre found in this serum is shown by the dashed lines (1:2 titre in HIV-uninf serum) and when diluted 2 fold with PBS (1:4 titre in HIV-uninf serum). (B) Opsonisation with anti-OAg antibodies purified from HIV-infected inhibitory serum and diluted to the titre found in HIV un-infected serum, and again diluted 2 fold with PBS. Dashed lines correspond to inhibition of killing when OAg antibodies from HIV-infected inhibitory sera are added at their source titre (1:2 titre in HIV-inf inhib serum) or diluted 2 fold with PBS (1:4 titre in HIV-inf inhib serum). Each panel represents one experiment with data from serum/antibodies from one African adult.

antibodies were able to induce cell-killing of *Salmonella* by acting as opsonins to stimulate phagocytosis and uptake of the bacteria. We know that *Salmonella* are effectively killed by blood cells when this antibody-depleted serum is supplemented with heat-inactivated human serum as an antibody source (see Chapter 4).

Salmonellae opsonised with 60% complement and 40% anti-OAg antibodies (added at the concentration found in the source serum) from HIV-uninfected serum were killed by blood cells ($1.5 \log_{10}$ kill after 180 min) whereas killing did not occur following opsonisation with complement and PBS ($1.5 \log_{10}$ growth) (Figure 5.12 A). However, this kill was reduced as the OAg antibodies were diluted, with loss of killing when purified OAg antibodies were diluted to 1:8 with PBS. When purified OAg antibodies from one of the HIV-infected inhibitory serum were used together with complement to opsonise the bacteria, then again killing was induced in contrast to opsonisation with complement in the absence of OAg antibodies (Figure 5.12 B), although this time killing was lost at a 1:4 dilution. This shows that both anti-OAg antibodies from HIV-uninfected and HIV-infected serum can opsonise *Salmonella* and induce cell-killing. Overall, the blood cell-mediated killing of *Salmonella* opsonised with pre-absorbed serum and OAg antibodies was reduced compared to that when heat-inactivated human serum, which contains no complement but a range of antibodies to *Salmonella* was used as the antibody source, suggesting that the OAg could be one of the targets of opsonic antibody but that antibody to other antigens also enhances killing of NTS.

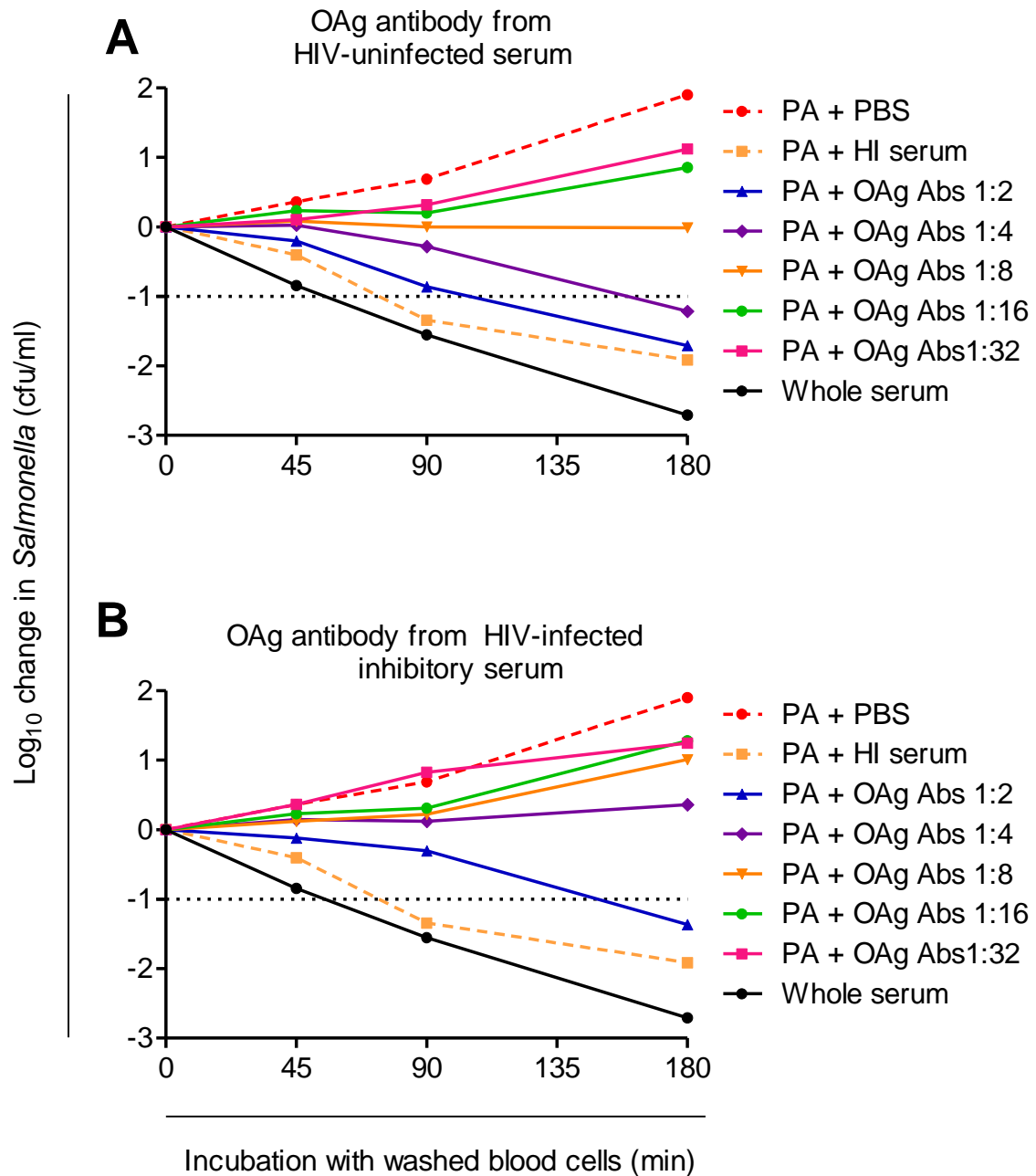


Figure 5.12 Blood cell killing of *S. Typhimurium* D23580 opsonised with human complement and diluted purified anti-OAg antibodies.

S. Typhimurium D23580 was opsonised with whole fresh HIV-uninfected serum (whole serum), 60% HIV-uninfected pre-absorbed serum as complement source (PA serum) and 40% PBS (PA + PBS), 60% pre-absorbed serum and heat-inactivated human serum as an antibody source (PA+HI serum) or 60% pre-absorbed serum and 40% purified anti-OAg antibodies from (A) HIV-uninfected African sera or (B) HIV-infected inhibitory sera. Purified anti-OAg antibodies were added at the titre found in the source serum (PA+OAg Abs 1:2) and then diluted 2 fold with PBS (PA + OAg Abs 1:4). Each panel represents one experiment using purified OAg antibodies from 2 African adults.

5.4 DISCUSSION

NTS infections have been associated with HIV since the onset of the AIDS epidemic (57). In Sub-Saharan Africa, high mortality rates are observed in HIV-infected adults who succumb to invasive NTS disease (30). Recurrent infections are common and the emergence of multi-drug resistant strains makes treatment with antibiotics challenging. Currently there is no available vaccine for NTS. An OAg conjugate is a potential vaccine candidate, but until now the functional activity of the OAg antibodies in cell-mediated immunity has not been investigated.

Through the use of a blood cell killing assay, and FACS-based phagocytosis and oxidative burst assays we were able to assess cell-mediated killing of NTS in healthy and HIV-infected African adults. These assays use human blood phagocytes rather than a cell-line and opsonises with 100% serum as a source of antibody and complement to make the assays as close to physiological conditions as possible. Using fresh human blood phagocytes from healthy HIV-uninfected adults allows us to focus on the effect of HIV-infected serum and humoral components of these sera and the function of antibody in these sera. For the blood cell killing assay, incubating opsonised *Salmonella* in RPMI rather than washed blood cells confirms that killing in this assay is cell-mediated.

Using the blood cell killing assay, we observed reduced killing by peripheral washed blood cells of *Salmonella* Typhimurium opsonised with serum from some HIV-infected adults. The inhibition was not observed in all HIV-infected adults, but rather in a group

of sera which had higher anti-*Salmonella* Typhimurium LPS IgG and IgA antibody titres as shown by ELISA, compared to healthy HIV-uninfected African adults.

All serum had functional complement (61), which rules out complement deficiency or failure to deposit complement on the surface of the bacteria as an explanation for reduced killing, and that impaired killing of *Salmonella* was observed only when opsonised with HIV-infected sera, which have higher serum anti-LPS titres, suggested that antibodies targeting the LPS of *Salmonella* are causing the inhibition. A *galE*-mutant of *S. Typhimurium* D23580 which lacks an OAg chain, underwent a 3 log₁₀ kill when opsonised with these HIV-infected inhibitory sera, suggesting that antibodies targeting the OAg of *S. Typhimurium* were responsible for inhibiting the killing of the wild-type isolate. Killing of *S. Typhimurium* D23580 opsonised with HIV-infected inhibitory serum which had anti-*S. Typhimurium* LPS antibodies removed was increased compared to whole untreated serum, but killing was not enhanced when antibodies to *S. Enteritidis* or *S. Minnesota* LPS, lipid A or to the core oligosaccharide were removed, providing further evidence for the involvement of anti-LPS antibodies in inhibiting killing of *S. Typhimurium* by peripheral blood cells.

The purification of anti- *S. Typhimurium* OAg antibodies by affinity chromatography (see Chapter 6) from HIV-uninfected and HIV-infected sera allowed the further characterisation of the role of anti-OAg antibodies in immunity to NTS. Inhibition of killing was observed when *Salmonellae* were opsonised with 50% serum and 50% OAg antibodies purified from 3 HIV-infected inhibitory sera, added at the source serum titre.

This inhibition could be removed by diluting the purified OAg antibody indicating a concentration-dependent inhibition of killing by OAg antibodies. OAg antibodies from these HIV-infected inhibitory sera were at a higher concentration than those from the other groups of sera, which suggests it is the titre, and not the source of anti-OAg antibodies which inhibits blood cell killing of *Salmonella*. Further support for this conclusion was the reduction in killing of *Salmonella* opsonised with 50% fresh serum and 50% OAg antibodies from a HIV-uninfected serum, concentrated to the elevated titre detected in one of the inhibitory serum and also, the increase in killing of *Salmonella* opsonised with 50% fresh serum and 50% OAg Abs purified from an HIV-infected inhibitory serum but diluted to the titre found in a HIV-infected non-inhibitory serum.

It has previously been shown that opsonic antibody plays an important role in immunity to NTS (145;151) but the identity of such opsonic antibody remains unclear. It is difficult to ascertain from the results discussed above whether anti-OAg antibodies can act in an opsonic manner and enhance killing of NTS by peripheral blood cells. To try to answer this question, we used human serum which has been pre-absorbed with whole bacteria to deplete it of all anti-*Salmonella* antibody, resulting in a human complement source which can be used to opsonise the bacteria (see Chapter 4). We know that when this antibody-depleted serum is supplemented with heat-inactivated human serum as an antibody source, killing is restored (see Chapter 4). When purified OAg antibodies, from either HIV-uninfected non-inhibitory or HIV-infected bactericidal inhibitory serum, were used as an antibody source, killing was restored and this killing

could be removed by dilution of the antibody source. Killing was not to the same level as that when heat-inactivated human serum containing antibodies to a range of antigens was used as the antibody source. This suggests that antibodies targeting the OAg could be one of the targets of opsonic antibody. To further examine this, the assays would need to be repeated with purified antibodies to other targets of *Salmonella*, such as the outer membrane proteins which have been shown to be important for protective immunity to *Salmonella* (61;160;182).

Further support for the role of opsonic antibodies to the OAg was also provided by the assays in which anti-LPS antibodies were removed from the serum through step-wise incubation with purified *S. Typhimurium* LPS. Removal of some LPS antibodies increased killing of *Salmonella* Typhimurium D23580, but opsonisation of *Salmonella* with serum which had undergone 12 cycles of pre-absorption reduced the killing, suggesting that removing too much anti-LPS antibody impaired killing, and that at the right concentration, anti-OAg antibodies can enhance killing of NTS, but at elevated titres found in some HIV-infected adults, they can inhibit killing.

We can speculate that the decreased phagocytic response of neutrophils and monocytes of bacteria opsonised with HIV-infected inhibitory serum compared to *Salmonella* opsonised with HIV-uninfected or HIV-infected non-inhibitory serum suggests that the inhibition observed for the blood cell killing isolates is phagocytosis mediated. The oxidative burst response of neutrophils and monocytes was not reduced in the presence of the HIV-infected serum which is surprising given that phagocytosis is a necessary

intermediate step in inducing the oxidative burst. Although it may be explained by a lower threshold required for the detection of oxidative burst activity than for phagocytosis in the assays used.

Complement as well as antibody is required for optimal phagocytosis and killing by blood cells (151). Therefore, we can hypothesise as previously proposed in relation to cell-free killing (61), that excess anti-LPS antibodies which bind to the OAg of LPS protruding from the surface of the bacteria could form a shield which diverts complement deposition away from the surface of the bacteria thus minimising the interaction between C3 and the C3 receptor on the phagocyte, and reducing uptake of the bacteria by lack of stimulation of the complement receptors on the phagocytes. This shield of LPS-antibodies could also block the access of other opsonic antibodies to their targets nearer the surface of the bacteria. For example, antibodies to the outer membrane proteins, in particular ompD (182), on the surface of the bacteria have been shown to be protective against *Salmonella* infection, and the excess presence of LPS-antibodies binding to the bacteria could block the access of these antibodies to the target antigens on the surface of the bacteria. These mechanisms have been suggested as reasons for impaired bactericidal activity in some HIV-infected sera (61). The observation that anti-LPS antibodies from healthy HIV-uninfected and HIV-infected adults can be opsonic and can induce killing at the right concentration but killing is enhanced if heat-inactivated serum is used, suggests that antibodies to other antigens as well as those to the OAg are opsonic. Alternatively, killing could be enhanced further when heat-inactivated serum is used as an antibody source compared to purified anti-

OAg antibodies, because complement fragments present in the heat-inactivated serum could be deposited on the bacteria.

A surprising observation was the significantly higher anti-LPS IgA titres observed in the HIV-infected inhibitory serum compared to the HIV-uninfected or HIV-infected non-inhibitory serum. IgA is known to have no opsonic activity (203;204) and has been previously shown to interfere with phagocytosis-mediated killing of pathogens (203). It is possible that IgA interferes with C3 fixation (203) or anti-LPS IgA could bind to the OAg and block binding of IgG, which is opsonic. Alternatively, both anti-LPS IgG and IgA could bind and IgA could reduce the binding of IgG to the FcR on the phagocyte by steric hindrance (205). To test whether elevated titres of anti-LPS IgA are responsible for inhibiting the killing of *Salmonellae* opsonised with HIV-infected inhibitory serum, these antibodies would need to be isolated from the sera. Unfortunately, due to limited serum volumes we have not been able to characterise the role of the various isotypes of anti-LPS antibody and study whether this is the inhibiting mechanism.

This work contributes another explanation as to the association between HIV and high incidence of NTS bacteraemia in HIV-infected African adults. A loss of Th17 cells from the gut mucosa leading to failure to maintain the epithelial barrier which causes easier dissemination of bacteria into the blood (60) , and cytokine dysregulation, in particular of IL-10, IL-12 and TNF α in advanced HIV infection (62), have so far been suggested as explanations for the association between HIV and NTS. Our group has also previously identified that high titres of anti-LPS antibodies block antibody-dependent

complement-mediated cell-free killing (61) by either blocking the access of antibodies to the outer membrane proteins and / or diverting complement deposition away from the surface of the membrane, thus preventing insertion of the membrane attack complex. Together, this work suggests that those HIV-infected adults who have a reduced ability to phagocytose and kill *Salmonella* by peripheral blood cells are also not able to kill *Salmonella* antibody via cell-free bactericidal killing. This could help explain the high incidence of bacteraemia observed in HIV-infected African adults as reduced phagocytosis of bacteria due to the presence of excess anti-LPS antibodies would result in the bacteria spending longer in the extracellular space where bactericidal anti-LPS antibodies are also unable to kill NTS, resulting in extracellular survival and development of bacteraemia. Phagocytosis of *Salmonella* opsonised with HIV-infected inhibitory serum was not completely absent, but rather significantly reduced compared to HIV-uninfected or non-inhibitory serum which signifies that some bacteria are taken up into the intracellular niche and can be killed by oxidative burst. However, recurrent NTS infections are observed in the context of HIV infection and it is likely that only a few of the bacteria which are phagocytosed need to resist killing via expression of SPI-1 genes in order to be able to reside in the intracellular niche, and together with cytokine dysregulation, establish recurrent infection.

There is currently no vaccine available for NTS, and given the evidence of protective immunity when prototype OAg conjugate vaccines have been tested in rabbits and mice (156-158), the development of polysaccharide conjugate vaccines for protection against NTS in humans is a promising avenue. However, immune responses in mouse models

and man can be very different and although this work suggests that anti-OAg antibodies are opsonic and induce clearance of *Salmonella* from the blood, in some humans the presence of excess anti-LPS antibodies inhibits both the uptake of bacteria into an intracellular location and bactericidal killing whilst in the extracellular space (61), which advises against the use of an OAg-conjugate as an effective vaccine for protection against NTS. It is important to further understand how HIV affects the immune response to NTS and to vaccinations, as HIV-infected adults as well as young children are increasingly susceptible to invasive NTS disease, and would consequently benefit the most from a vaccine. The other targets of protective opsonic antibody need to be identified to further inform vaccine development.

5.5 CONCLUSIONS

Killing of an invasive isolate of NTS, *Salmonella* Typhimurium D23580, by peripheral washed blood cells was impaired when bacteria were opsonised with sera from some HIV-infected adults. A high titre of anti- *S. Typhimurium* LPS antibody in these sera appears to inhibit the killing of *S. Typhimurium* by reducing phagocytosis and clearance of the *Salmonella* by peripheral adult blood cells. Pre-absorption of anti-LPS antibodies restored killing, and opsonisation of *Salmonellae* with high titres of purified anti-OAg antibodies inhibited killing of the bacteria by blood cells. This inhibition is concentration dependent and at the titre found in healthy Malawian adults, the anti-OAg antibodies can be opsonic and induce cell-mediated killing of *Salmonella*. In conclusion, this work argues for and against production of an OAg vaccine, as although anti-OAg

antibodies can be opsonic and induce cell-mediated killing of bacteria, at high titres they appear to block both bactericidal and opsonic mechanisms of immunity to NTS.

CHAPTER 6. PURIFICATION OF ANTIBODIES TO O-ANTIGEN OF S. TYPHIMURIUM FROM HUMAN SERUM BY AFFINITY CHROMATOGRAPHY

6.1 INTRODUCTION

There is an imperative need for a vaccine which will provide protection against invasive nontyphoidal *Salmonella* disease, particularly in Africa. Given the evidence that antibodies against the O-antigen (OAg) of lipopolysaccharide (LPS) of *Salmonella* provide protection against challenge in animal studies (156;157), then designing a vaccine which will increase production of antibodies to this antigen is a potential option. However, recent studies in HIV-infected adults (61) suggest that an excess of antibody targeting the OAg of *S. Typhimurium* actually impairs immunity to NTS. In order to further characterise the role of these antibodies in cell-mediated immunity to NTS, particularly in the context of HIV-infected African adults (Chapter 5), we needed to isolate them from human serum.

Monoclonal and polyclonal antibodies are conventionally purified by affinity chromatography (206;207) using the highly specific nature of the interaction between antigen and antibody. The antigen is chemically immobilized to a solid support under conditions that retain its antibody-binding capacity. When serum is passed through a column containing the antigen attached to the support, only those antibodies with specific affinity for the antigen are bound. After washing, the bound antibodies are

eluted, thereby purifying them from the original sample. Although this method for recovering antibodies is potentially selective, rapid and simple, the recovery is often low (208). Appropriate conditions need to be determined to permit efficient purification of the desired antibodies without altering their native structure (209).

Salmonella LPS consists of lipid A, which anchors LPS into the bacterial membrane, linked to the 3-deoxy-D-manno-octulosonic acid (KDO) terminus of the conserved core oligosaccharide region, and the OAg chain is attached to the core oligosaccharide. The OAg chain is the immunodominant portion and extends as a repeating polymer from the end of the core region (210). The composition of the OAg chain varies between serovars. In *S. Typhimurium*, the OAg repeat (O:4,5) consists of a trisaccharide backbone, with a branch of abequose, usually O-acetylated at C-2, which confers serogroup specificity (factor 4,5) (Figure 6.1 A) (211).

Since LPS is an endotoxin, LPS detoxification is usually performed by acetic acid hydrolysis or by hydrazinolysis (212), with the former commonly preferred as it retains the O-acetyl groups along the OAg chain. Acid hydrolysis cleaves the labile linkage between Lipid A and KDO leaving the OAg chain attached to the core region (Figure 6.1 A). Many approaches have been used to bind LPS or detoxified OAg from various bacteria to resins for use in affinity purification and, despite the high toxicity, CNBr-activated resin has been the most commonly used (213;214). Girard and Goichot attached LPS to an aminohexyl-Sepharose resin by activation with benzoquinone (215), while Fox and Hechemy tested epoxy-activated resin for the attachment of *Escherichia*

coli LPS (216). The bound LPS showed low antibody binding capacity, which may be due to limited availability of antibody-binding sites on LPS resulting from steric hindrance and/or partial hydrolysis as a result of the alkaline pH used for the LPS coupling to the resin.

For anti-NTS OAg antibody purification, affinity columns are not commercially available, and as a consequence we had to develop our own process for purifying antibodies to this antigen. We compared two alternative strategies for inserting hydrazide groups into the OAg of *S. Typhimurium* prior to linking to commercially available N-hydroxysuccinamide (NHS)-Sepharose resin. The resulting affinity columns were tested for their ability to isolate antibodies from human serum against the OAg and core of LPS from *S. Typhimurium* D23580, a characteristic invasive African isolate of NTS (217).

6.2 OBJECTIVES

The aim of this project was to develop a method to isolate antibodies specific to the OAg of *Salmonella* Typhimurium from human serum. Since commercial affinity columns are not available, we tested two different chemistries to identify the best method for linking the OAg and oligosaccharide core from *S. Typhimurium* to the resin to produce reusable affinity columns. Antigen binding and elution buffers were optimised to ensure the highest yield of functional specific antibodies were obtained, which could be used for further assays to identify their role in cell-mediated immunity to NTS.

6.3 RESULTS

6.3.1 PURIFICATION OF O-ANTIGEN

The LPS of *Salmonella* comprises lipid A, which is attached to a core oligosaccharide, which in turn is linked to an OAg chain composed of repeating sugar units (Figure 6.1). In order to purify antibodies to the OAg of *Salmonella*, we first needed to separate OAg and the core oligosaccharide from lipid A so that it can be activated and attached to the resin. *Salmonella* Typhimurium D23580, a characteristic invasive Malawian NTS isolate was used as a source of LPS. The OAg was purified following a method developed at Novartis Vaccines Institute for Global Health (NVGH), Siena, Italy. The purification and subsequent characterisation were performed by Francesca Micoli and Massimiliano Gavini at NVGH. The purification uses acetic acid hydrolysis of *Salmonella* fermentation culture to remove lipid A and release OAg into the supernatant, which can then be recovered by centrifugation, and impurities such as proteins and nucleic acids removed by filtration. This method is preferred to other methods that require hot phenol LPS extraction and LPS detoxification (158;202;212).

The resulting purified OAg attached to the oligosaccharide core contained 0.4% protein and 0.15% nucleic acid as determined by micro BCA. The chromogenic kinetic limulus amoebocyte lysate assay was used to measure the endotoxin level which was <0.01 UI/μg. HPLC-SEC analysis was used to estimate the molecular size distribution of OAg populations and identified the presence of two main populations with different average MW, with an average number of repeating units per OAg chain of 71 and 25. KDO

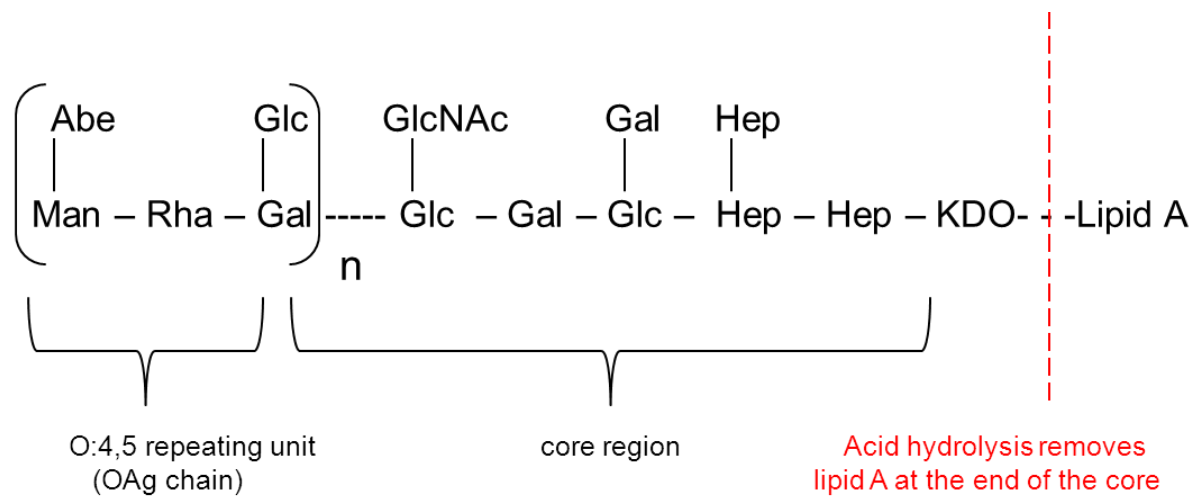


Figure 6.1 Structure of *S. Typhimurium* LPS.

The structure of the *S. Typhimurium* OAg chain linked to the core region after acetic acid hydrolysis. Acid hydrolysis removes Lipid A leaving the OAg chain attached to the core region and the KDO group of the core region exposed.

quantification using HPLC-SEC confirmed the presence of one KDO per OAg chain. A TNBS assay which is used for total NH₂ groups quantification, confirmed that the purified OAg contained 24.1% NH₂ groups which can react with adipic dihydrazide (ADH) to form a stable bond between linker and antigen.

6.3.2 ACTIVATION OF PURIFIED O-ANTIGEN WITH ADH AND COUPLING TO NHS-SEPHAROSE COLUMN

To produce an affinity column the antigen needs to be linked to the resin to form a surface to which the antibodies in the serum can bind and be eluted from. Since the purified OAg and core cannot bind directly to the resin, it first needs to react with another compound which can act as a linker between the antigen and resin and facilitate binding of OAg to the commercial NHS -Sepharose resin. We used adipic dihydrazide (ADH) as a linker as it is a symmetrical molecule with two hydrazide groups: one group can react with the ketone or aldehyde groups of the OAg and the other is able to react with N-hydroxy succinamide groups on the resin thus linking the antigen to the resin.

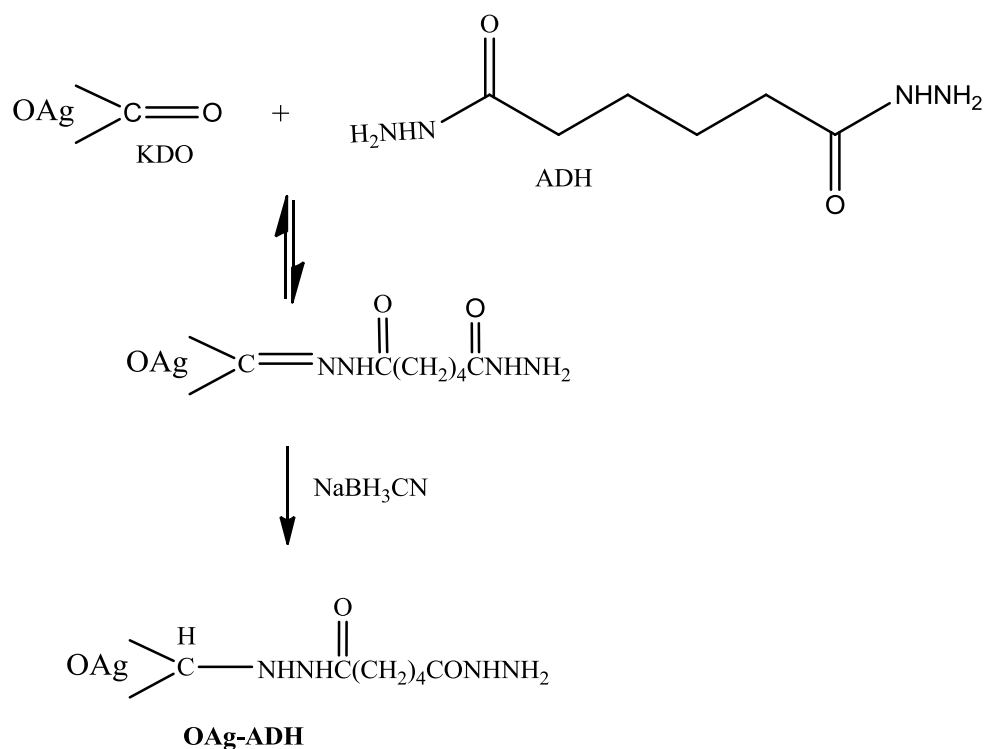
Two different chemistries were used for inserting reactive hydrazide groups into the OAg prior to linking to commercially-available NHS-Sepharose. For the first method, the carbonyl group of the KDO sugar at the end of the core region which was exposed after acetic acid hydrolysis and removal of lipid A, was linked through reductive amination to the NH₂ group of one ADH molecule, with the formation of an amine linkage. This

produced OAg-ADH, with one ADH molecule attached at the end of the core region of the OAg chain, thus not modifying the OAg chain (Figure 6.2 A). For the second method, OAg underwent an oxidative step with sodium metaperiodate prior to activation with ADH. The oxidation with sodium metaperiodate produces aldehyde groups along the length of the OAg chain which can then react with the NH₂ group of ADH by reductive amination (Figure 6.2 B). The resulting OAgoxADH chain is modified with multiple ADH linkers introduced along the polysaccharide chain. Once the ADH had been linked to the OAg to produce OAgADH or OAgoxADH, the activated OAg-core was applied to commercial NHS-Sepharose columns to form an affinity matrix. In theory, the presence of more than one ADH linker per OAg chain in OAgoxADH could favour the OAg binding to the NHS-Sepharose, but in fact 3.7 mg of OAgoxADH and 4.3 mg of OAg-ADH were linked per ml of resin for each column. The resulting affinity columns will, in theory bind antibodies specific to the O-antigen and core polysaccharide from human serum, allowing purification of antibodies to the core as well as the O-antigen.

6.3.3 TESTING ANTIGENIC INTEGRITY OF ACTIVATED O-ANTIGEN

To determine if the hydrolysis and subsequent activation of OAg with ADH had impaired the antigenic structure of the OAg, the two affinity columns we had prepared were tested using a commercial preparation of purified polyclonal rabbit anti-*Salmonella* Typhimurium O:4,5 antibodies. 300 µl of the O:4,5 antibody preparation (with an antibody concentration corresponding to 1666 ELISA units) was applied to an NHS-Sepharose column with 4.3 mg OAgADH linked and also to an NHS-Sepharose

A



B

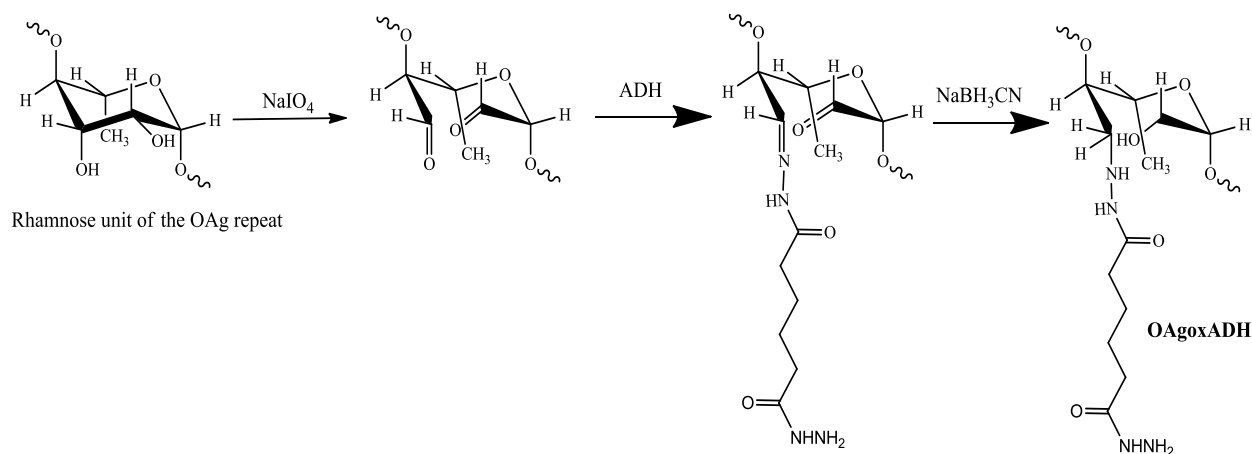


Figure 6.2 Reaction schemes for production of activated OAg.

Reaction scheme for OAg-ADH: the ketone group of the terminus KDO is linked to ADH by reductive amination (A). Reaction scheme for OAgoxADH: diol groups are oxidised to aldehyde groups by NaIO_4 . These aldehyde groups can then react with ADH by reductive amination (B).

column with 3.7 mg of OAgoxADH attached. The recovery of antibody in the flow through, washes (containing unbound antibody) and eluates were quantified by ELISA using plates coated with purified *S. Typhimurium* LPS. 92% of the applied antibodies bound to the OAgADH column (Figure 6.3 A) and 96% bound to the OAgoxADH column (Figure 6.3 B), with the remaining applied antibodies detected in the flow through or wash fractions. 89% of the bound anti-*Salmonella* antibodies were successfully eluted from the column using 0.1M glycine, 0.1 M NaCl pH 3 as the elution buffer. 90% of the antibodies which were bound to the OAgoxADH column were also eluted using the same buffer. The high percentages of purified anti-*Salmonella* Typhimurium rabbit antibodies which bound to the resins confirmed that the purification and activation of OAg had not affected the antigenic structure of the OAg and that the prepared columns are suitable for antibody purification.

6.3.4 PURIFICATION OF ANTIBODIES FROM HUMAN SERUM

The main aim of this chapter is to purify antibodies from serum taken from HIV-uninfected and HIV-infected African adults. Therefore, once we had confirmed the functional antigenic integrity of both activated OAg-NHS-sepharose columns, we tried to purify anti-OAg antibodies from human serum. Serum from a healthy, HIV-uninfected adult was treated with ammonium sulphate to precipitate the proteins and to reduce the presence of contaminants which could interfere with the interactions between the immobilised OAg and the corresponding polyclonal antibodies.

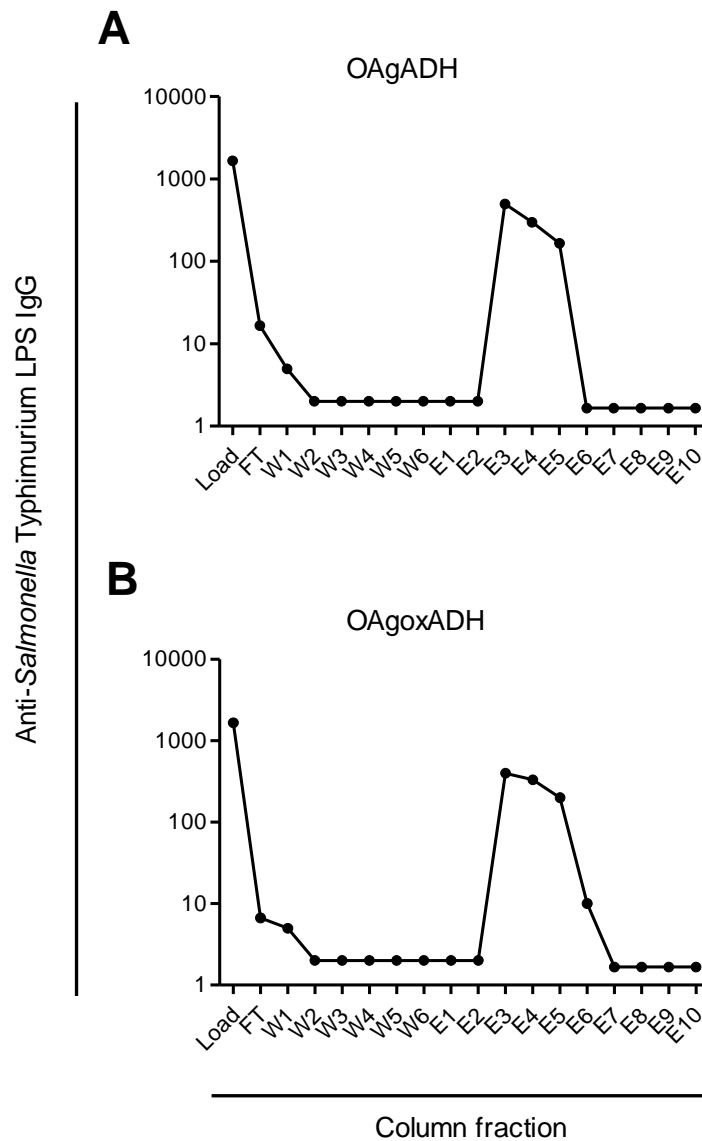


Figure 6.3 Activated OAg from *S. Typhimurium* D23580 coupled to NHS-Sepharose can reversibly bind antibody.

ELISA profile for the binding and elution of anti-*Salmonella* Typhimurium O:4,5 IgG antibody from an OAg-ADH (A) and OAgoxADH NHS-Sepharose column (B). 300 μ l of anti-*Salmonella* Typhimurium O:4,5 rabbit antiserum (Biorad), with an antibody concentration corresponding to 1666 ELISA units, was applied to the column (load). Flow through (FT) containing unbound antibody was collected, and the column washed with 6 ml PBS (W1-3), followed by 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6). Antibody was eluted with 5 ml 0.1 M glycine, 0.1 M NaCl pH 3 in 500 μ l fractions (E1-10). All fractions were collected and the concentration of anti-LPS IgG antibody in each fraction was tested by ELISA. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Data are representative of two experiments.

300 µl of the resulting serum protein solution (with an antibody concentration corresponding to 1000 ELISA units) were applied to the 2 columns. More than 75% of the antibodies in the applied serum protein solution bound to the each resin as shown by the low signal in both ELISAs for the flow through and wash fractions (Figure 6.4 A and B). Bound antibodies were again eluted with 0.1 M glycine, 0.1 M NaCl pH 3 and immediately neutralised with 2 M Tris pH 9. For the OAgADH column (Figure 6.4 A), eluting under these conditions, only 14% of the bound antibodies were detected in the eluate fractions in the ELISA. For the OAgoxADH column, the recovery of purified antibody decreased to 2% (Figure 6.4 B). It is difficult to ascertain if the modification of the OAg chain due to the attachment of multiple ADH molecules causes the observed decrease in antibody recovery as although the OAg chain modifications do not appear to affect the binding of antibody to the antigen, they may affect the release of antibody.

To ensure that the purified antibodies we detected in the ELISA using plates coated with LPS were specifically against the OAg rather than other LPS components such as the lipid A or core oligosaccharide, we repeated the ELISA using plates coated with OAg purified from *S. Typhimurium* D23580. The same titres were obtained for the column fractions regardless of the antigen used to coat the plates, confirming that the antibody we were detecting were specific for the OAg. Since LPS is commercially available, plates for all the following ELISAs were coated using LPS rather than OAg.

Despite the apparent binding of over 75% of human anti-LPS antibody to both columns, the recovery of these antibodies was very low, particularly with OAgoxADH. Given that

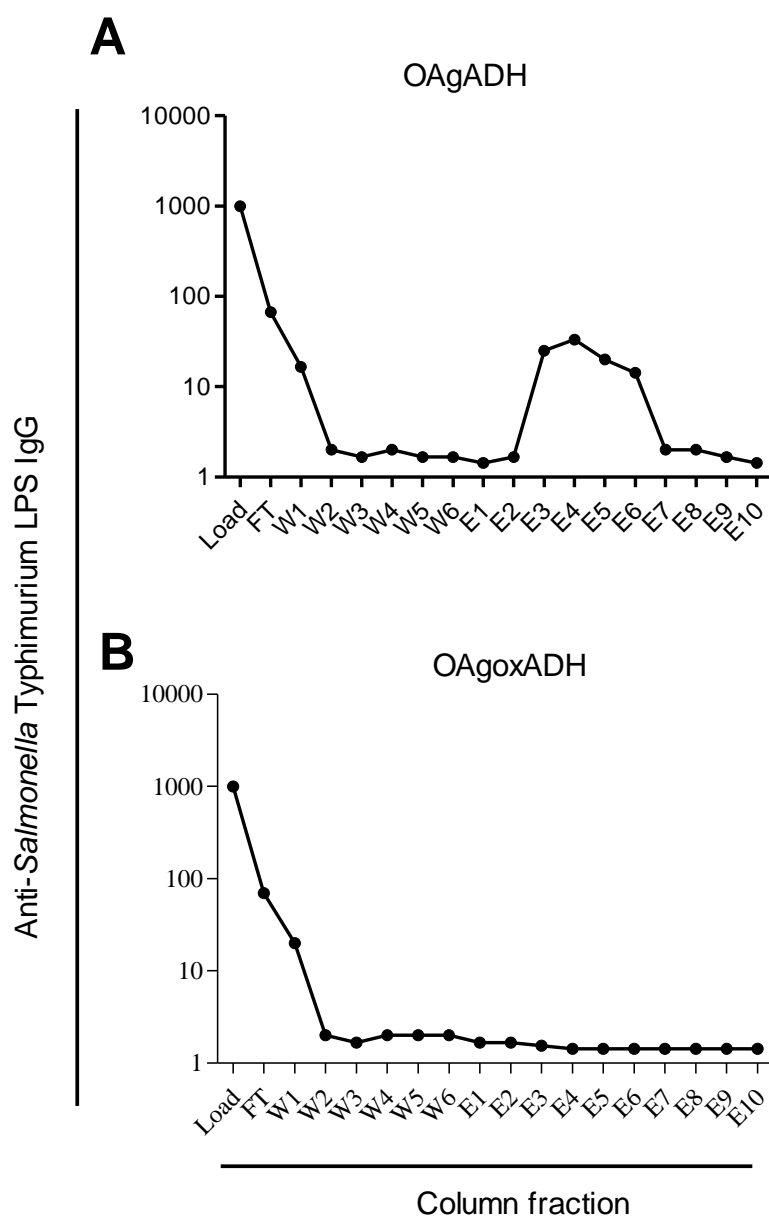


Figure 6.4 Activated OAg from *S. Typhimurium* D23580 coupled to NHS-Sepharose can reversibly bind human polyclonal antibodies.

ELISA profile for the binding and elution of human anti-*Salmonella* Typhimurium antibodies from an (A) OAg-ADH and (B) OAgoxADH NHS-Sepharose column. 300 μ l of ammonium sulphate-precipitated human serum proteins with an anti-*Salmonella* Typhimurium antibody concentration corresponding to 1000 ELISA unites was applied to the column (load). Flow through (FT) containing unbound antibody was collected, and the column washed with 6 ml PBS (W1-3), followed by 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6). Bound antibodies were eluted with 5 ml 0.1 M glycine, 0.1 M NaCl pH 3 in 500 μ l fractions (E1-10). All fractions were collected and the concentration of anti-LPS IgG antibody in each fraction was tested by ELISA. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Each panel represents data using one column and is representative of 3 experiments.

the binding capacity of the OAgADH to NHS-Sepharose resin was similar to OAgoxADH, that it is easier to prepare as only one step is required for its synthesis compared to two steps for the OAgoxADH, and that the yield of purified antibody, although still low, is higher than that for the OAgoxADH, OAgADH was selected as the most suitable activated antigen for use for affinity purification of OAg antibodies.

6.3.5 OPTIMISING BINDING OF ANTIBODIES TO OAGADH-NHS-SEPHAROSE COLUMNS

Ammonium sulphate precipitation is commonly used to concentrate proteins within the serum although antibody is lost during this step. We investigated whether the binding of antibodies from non-precipitated whole serum was increased compared to the binding of antibodies in ammonium sulphate protein precipitate solution. When whole serum (antibody concentration corresponding to 1425 ELISA units) which had not undergone ammonium sulphate precipitation, was applied to the OAg-ADH column (Figure 6.5 A), binding of antibody to the column was reduced, with only 50% of the applied antibodies binding to the resin. 15% of applied antibodies were lost in the flow through and 35% lost in the wash fractions. The recovery of purified antibodies was 11%. We decided to use ammonium sulphate pre-treatment of the serum before applying the serum to the column as although antibodies are lost during the precipitation step, the overall binding of antibody was higher (75%, Figure 6.3 A).

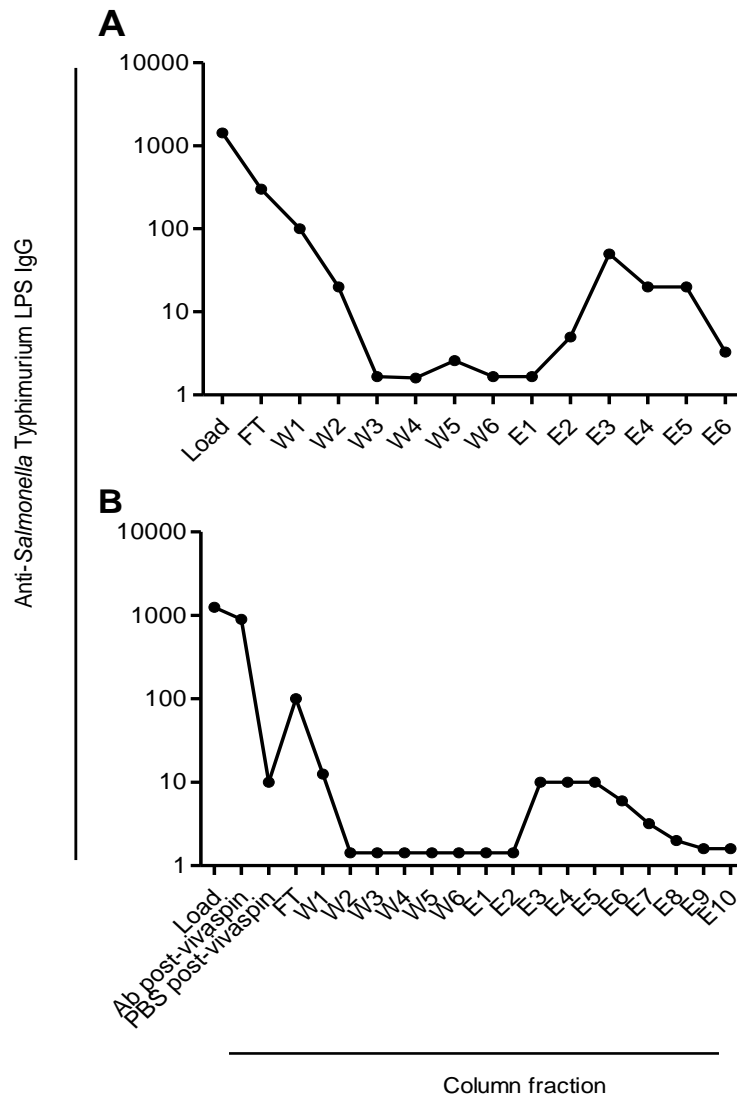


Figure 6.5 Decreased binding of anti-OAg antibodies from non-precipitated human serum or from Vivaspin treated human serum preparation.

300 μ l of whole human serum (with an anti-OAg *Salmonella* Typhimurium antibody concentration corresponding to 1425 ELISA units), which had not been treated with ammonium sulphate prior to applying to the column, was loaded onto an OAgADH column (A). Flow through (FT) containing unbound antibody was collected, and the column washed with 6 ml PBS (W1-3), followed by 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6). Bound antibodies were eluted with 3 ml 0.1 M glycine, 0.1 M NaCl pH 3 and collected in 500 μ l fractions (E1-6). All fractions were collected and the concentration of anti-LPS IgG antibody in each fraction was tested by ELISA. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. (B) 300 μ l of ammonium sulphate precipitated human serum preparation (Load – with an antibody concentration of 1250 ELISA units) was passed through a 10 kDa Vivaspin column to remove small impurities prior to loading on an OAgADH column. The solution post-Vivaspin treatment was applied to the column (Ab post-Vivaspin, 900 ELISA units), and flow through, washes and eluates collected as described above. 50 μ l of the PBS solution which came through the Vivaspin column (PBS post-Vivaspin) was also tested in the ELISA to detect if antibody was lost during centrifugation. Data are from one experiment.

It is likely that the increased binding of antibodies in the protein preparation compared to whole serum is a consequence of removing impurities within the serum which can interfere with the antigen-antibody interaction. In an attempt to further remove small impurities from the protein preparation precipitated from human serum prior to applying to the column, the ammonium sulphate antibody precipitate was centrifuged using Vivaspin columns with a 10 kDa molecular weight cut-off. In theory, the resulting protein solution will contain less impurities and the binding of antibody to the OAg may be increased.

To verify if any antibody was lost during the Vivaspin pre-treatment, we quantified the anti-LPS antibody titre in the serum before and after the Vivaspin treatment using an ELISA. A protein preparation from human serum, corresponding to 1250 ELISA units was applied to the Vivaspin column, and 900 ELISA units were quantified post-treatment, confirming that 28% of the antibodies applied to the Vivaspin column were lost during this process (Figure 6.5 B). Antibodies were detected in the PBS solution which passed through the Vivaspin column, which accounts for some of the lost antibody. Some antibody probably remained attached to the membrane within the spin column.

The OAgADH column was loaded with 300 µl of the protein precipitate which had undergone Vivaspin treatment (with an antibody concentration corresponding to 900 ELISA units) and incubated overnight at 4°C. The column was washed to remove any

unbound antibody and 8% of the bound antibodies were eluted with 0.1 M glycine, 0.1 M NaCl, pH 3. Given that the binding of antibody was around 75%, which was the same as the binding for antibodies which had not undergone Vivaspin pre-treatment (Figure 6.3 A), that the yield was not enhanced, and that 28% of the antibodies were lost during the Vivaspin pre-treatment, then this is not a viable option.

6.3.6 COMPARING ELUTION OF ANTIBODIES FROM OAgADH COLUMN WITH GLYCINE AT FOUR DIFFERENT PH AS THE ELUTION BUFFER

Although the recovery of anti-*Salmonella* Typhimurium rabbit antibodies from the OAgADH column was high using 0.1 M glycine, 0.1 M NaCl pH 3, the recovery of human anti-*Salmonella* antibodies under these conditions was low. Glycine at acidic pH is commonly used as an elution buffer (209). With the aim of improving the recovery of antibody from the OAgADH column, we tested 0.1M glycine, 0.1 M NaCl at decreasing pH; pH 3, pH 2.8, pH 2.6 and pH 2.4.

Four OAg-ADH NHS Sepharose columns were prepared, each with 3.5 mg of OAg-ADH linked to the resin, using the same batch of purified activated OAg. Precipitated human serum proteins from the same donor as in the previous experiments (with an antibody concentration corresponding to 1300 ELISA units), were applied to each column. The amount of antibody lost in the flow through and washes and detected in the eluates was quantified using an ELISA. The relative amount detected in the flow through and wash fractions was low, and comparable for all four columns confirming that antibody had

bound to all four columns (Figure 6.6 A-D). A different elution buffer was tested in a different column and the recovery of antibody compared. Elution with 0.1 M glycine, 0.1 M NaCl pH 3 eluted 9% of the bound antibody (Figure 6.6 A), pH 2.8 removed 16% of the bound antibody (Figure 6.6 B), 12% was recovered in eluates using the buffer at pH 2.6 (Figure 6.6 C) and pH 2.4 was optimal and eluted 26% of the bound antibodies (Figure 6.6 D).

6.3.7 COMPARING ELUTION OF ANTIBODIES FROM OAGADH COLUMN WITH FOUR DIFFERENT ELUTION BUFFERS

The ideal elution buffer efficiently releases the bound antibody, without causing lasting damage to the native structure of the protein. Depending on the interactions involved between the antibody, antigen and resin, buffers with different elution mechanisms are required. The use of 20% ethanol, an organic solvent, 100 mM Tris pH 9 as an alkaline buffer, 8 M urea as a denaturant and 4 M MgCl₂ which raises the ionic strength of the solvent, with an accompanying weak chaotropic effect, were studied to identify if a buffer using an alternative elution mechanism to glycine at acidic pH could be more effective and increase the recovery of purified antibodies.

Four OAg-ADH NHS columns were prepared, each with 3.5 mg from the same batch of activated OAg-ADH linked to the resin. Precipitated human serum proteins (with an antibody concentration corresponding to 1300 ELISA units), were applied to each column. The relative amount of unbound antibodies detected in the flow through and

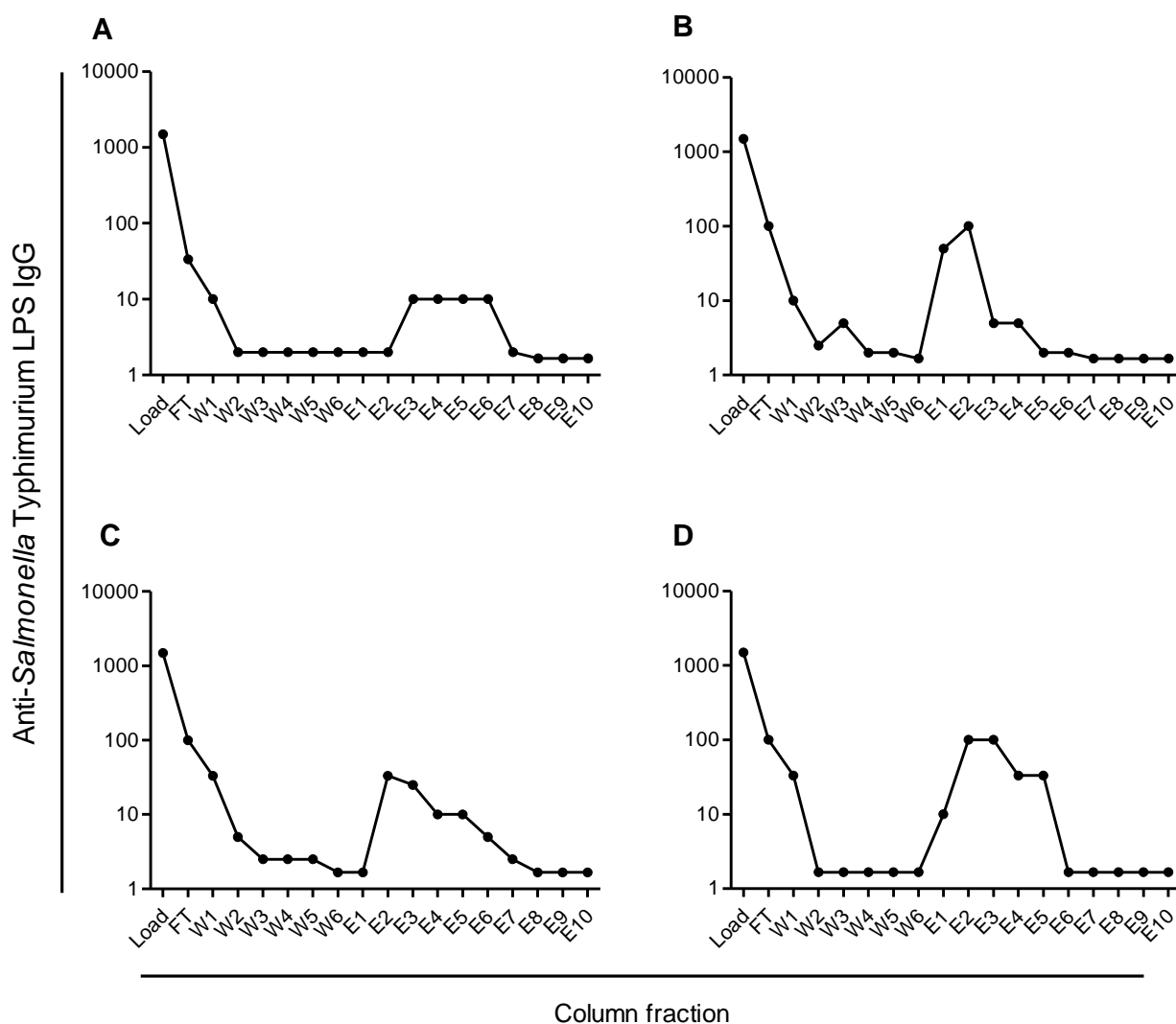


Figure 6.6 Elution of anti-OAg antibodies from human serum using glycine with a decreasing pH.

Four OAgADH-NHS-Sepharose columns were loaded with 300 μ l of ammonium sulfate-precipitated human serum proteins with an anti-*Salmonella* Typhimurium antibody concentration corresponding to 1300 ELISA units (load) and the flow through (FT) collected. Each column was washed with 6 ml PBS (W1-3), 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6). 5 ml of different elution buffers were passed through each column: (A) 0.1 M glycine, 0.1 M NaCl pH 3, (B) 0.1 M glycine, 0.1 M NaCl pH 2.8, (C) 0.1 M glycine, 0.1 M NaCl pH 2.6 and (D) 0.1 M glycine, 0.1 M NaCl pH 2.4. Eluates were collected in 500 μ l fractions (E1-10). Concentration of anti-OAg IgG antibody in each fraction was tested by ELISA using LPS coated plates. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Each panel represents data using one column and is representative of 3 experiments.

wash fractions was very low and comparable for all four columns (Figure 6.7 A-D). 4 M MgCl_2 in 10 mM Tris pH 9 was the most effective of the four elution buffers tested, eluting 18% of the bound antibody (Figure 6.7 B). Ethanol (Figure 6.7 A) and urea (Figure 6.7 D) removed 7% of the bound antibodies from the column, and 100 mM Tris pH 9 removed only 1% of the bound antibody (Figure 6.7 C). None of the buffers tested were as effective as 0.1 M glycine, 0.1 M NaCl pH 2.4 in releasing bound anti-OAg antibodies from the OAgADH columns.

6.3.8 SEQUENTIAL ELUTION OF ANTI-O-ANTIGEN ANTIBODIES

Polyclonal antibodies can have different affinities for the same antigen and as a consequence different elution buffers may be required to disrupt the antigen-antibody interaction. Although we previously identified that glycine at pH 2.4 eluted the most bound antibody, MgCl_2 also removed 18% of the antibody which was bound to another OAg-ADH column. Given that different antibodies may require different elution mechanisms, we determined if the recovery of human polyclonal anti-OAg antibodies could be increased by sequentially passing these two buffers through a column with anti-OAg antibodies attached. One OAg-ADH column was loaded with 300 μl precipitated human serum proteins (antibody concentration corresponding to 1300 ELISA units) and the amount of antibody in the flow through, washes and eluates quantified using an ELISA. Bound antibodies were sequentially eluted with 10 ml 0.1 M glycine, 0.1 M NaCl pH2.4, and 10 ml 4 M MgCl_2 in 10 mM Tris pH 7 with 6 ml PBS passed through the column between the two buffers. 28% of the bound antibody was

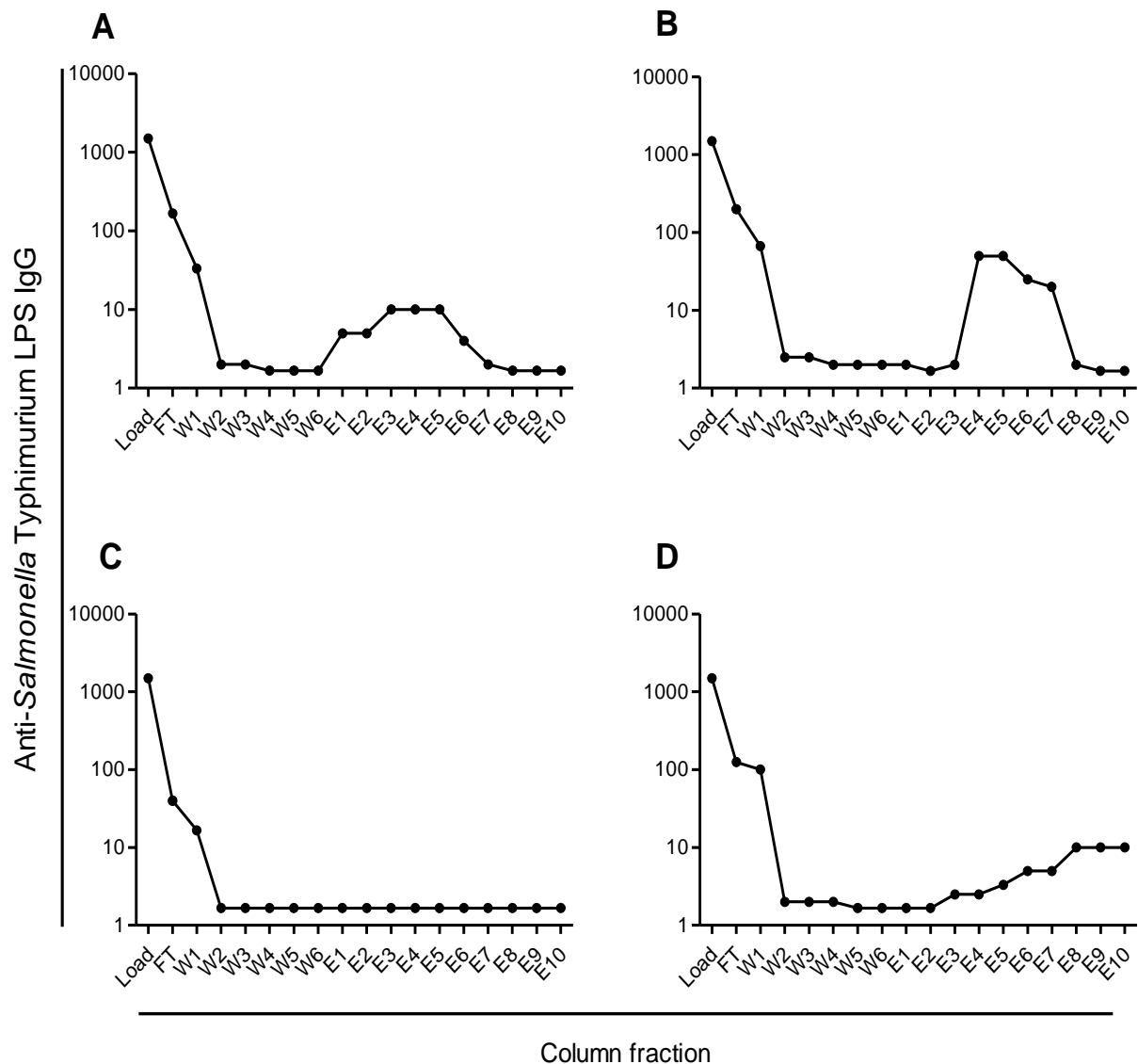


Figure 6.7 Ability of different buffers to elute bound anti-OAg IgG antibodies from human or rabbit serum.

Four OAgADH-NHS-Sepharose columns were loaded with 300µl of ammonium sulfate-precipitated human serum proteins with an anti-*Salmonella* Typhimurium antibody concentration corresponding to 1300 ELISA units (load) and the flow through (FT) collected. Each column was washed with 6 ml PBS (W1-3), 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6). 5 ml of different elution buffers were passed through each column: (A) 20% ethanol, (B) 4 M MgCl₂ in 10 mM Tris base pH 7, (C) 100 mM Tris base pH 9, and (D) 8 M urea pH 7 and the eluates collected and neutralised if required (E1-10). Concentration of anti-OAg IgG antibody in each fraction was tested by ELISA using LPS coated plates. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Each panel represents data using one column and is representative of 3 experiments.

removed with 0.1 M glycine, 0.1 M NaCl pH 2.4 (Figure 6.8 B), but no further antibody was removed when MgCl₂ was passed through the column, suggesting that when the two buffers were tested with separate columns, they were removing the same population of low affinity antibody.

300 µl of commercial rabbit anti-O:4,5 *Salmonella* Typhimurium antibodies (antibody concentration corresponding to 1666 ELISA units) was applied to an OAg-ADH column, and sequential elution repeated, this time using all four buffers which have different elution mechanisms; MgCl₂, ethanol, urea and glycine pH 2.4 (Figure 6.8 A). Eluting with 3 ml 4 M MgCl₂ in 10 mM Tris pH 7, only removed 44% of the bound antibodies. No antibodies were removed using 3 ml 8 M urea or 3 ml 20% ethanol, but elution with 3 ml 0.1 M glycine, 0.1 M NaCl pH 2.4, a further 31% of the bound antibodies were recovered.

6.3.9 DECREASING THE AMOUNT OF ACTIVATED O-ANTIGEN COUPLED TO NHS-SEPHAROSE INCREASES THE RECOVERY OF PURIFIED ANTIBODY

Despite using optimal binding and elution conditions, the recovery of purified antibody still remained low. One explanation for the low recovery of antibody could be due to an excess of OAg in the column, which could form multiple high and low affinity sites. Antibody from low affinity sites is more easily removed as the interaction between the antigen and the resin is easier to disrupt. Therefore, if the number of overall binding

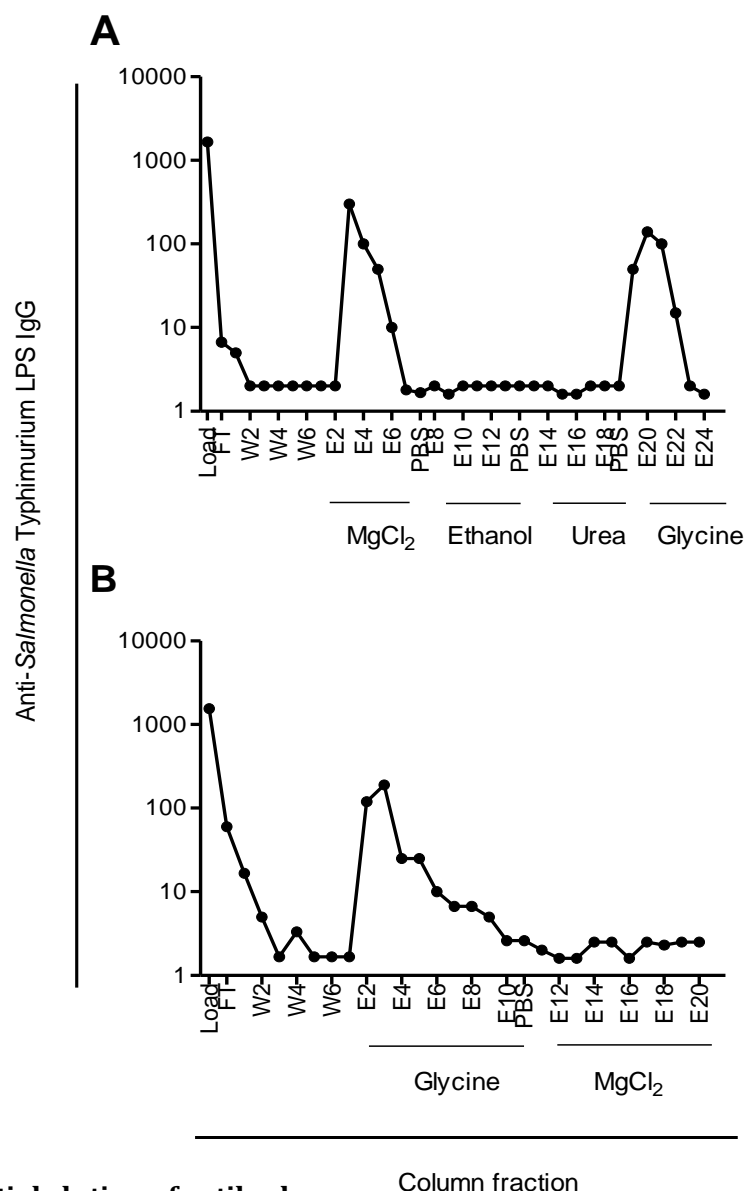


Figure 6.8 Sequential elution of antibody.

(A) 300 μ l of anti-*Salmonella* Typhimurium 0:4,5 rabbit antiserum (Biorad), with an antibody concentration corresponding to 1666 ELISA units, was applied to the OAg-ADH column (load), flow through (FT) containing unbound antibody collected, and the column washed with 6ml PBS (W1-3), followed by 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6). Bound antibody was sequentially eluted with 3 ml 4 M MgCl₂ in 10 mM Tris base pH 7; then 3 ml 20% ethanol; 3 ml 8 M urea pH 7; and 3 ml 0.1 M glycine, 0.1 M NaCl pH 3 with 6 ml PBS passed through the column between buffers. Eluates were collected in 500 μ l fractions (E1-10). (B) OAg-ADH column was loaded with 300 μ l precipitated human serum with an IgG antibody concentration corresponding to 1250 ELISA units, and bound antibody was eluted with 10 ml 0.1 M glycine, 0.1 M NaCl pH 3, 6 ml PBS followed by 10 ml 4 M MgCl₂ in 10 mM Tris base pH 7. Concentration of anti-OAg IgG antibody in each fraction was tested by ELISA using LPS coated plates. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Each panel represents data using one column and is representative of 3 experiments.

sites is reduced, in particular the number of high affinity binding sites, then maybe more antibodies will bind to the low affinity sites where they can be more easily eluted.

To test this hypothesis, we investigated how varying the ratio of OAg coupled to NHS-Sepharose in the column affects the recovery of purified antibodies. 3.5 mg OAg-ADH per ml of resin was immobilised on the columns used for previous extractions, and we investigated the effect of decreasing the amount of OAgADH immobilised on the column to 1 mg and 0.5 mg per ml of resin. Equal amounts of precipitated human serum proteins were applied to three new columns which had 3.5 mg, 1 mg or 0.5 mg of OAgADH linked to NHS-Sepharose and the amount of antibody linked to the column quantified by detecting the amount of antibody detected in the flow through and washes. 80% of the loaded antibodies were retained for each column, regardless of the amount of OAg linked to the matrix (Figure 6.9 A-C). 0.1 M glycine, 0.1 M NaCl pH 2.4, which we had verified was the most effective elution buffer, was used to elute bound antibody. 26% of the antibody bound to the 3.5 mg OAgADH column were eluted (Figure 6.9 A), and the recovery of purified antibody increased to 51% for the column with 1 mg OAgADH attached to the resin (Figure 6.9 B). The yield decreased to 19% for the column with the lowest amount of OAgADH linked (Figure 6.9 C). This indicates that the antibody-antigen ratio is an important factor for determining reversible antibody interactions.

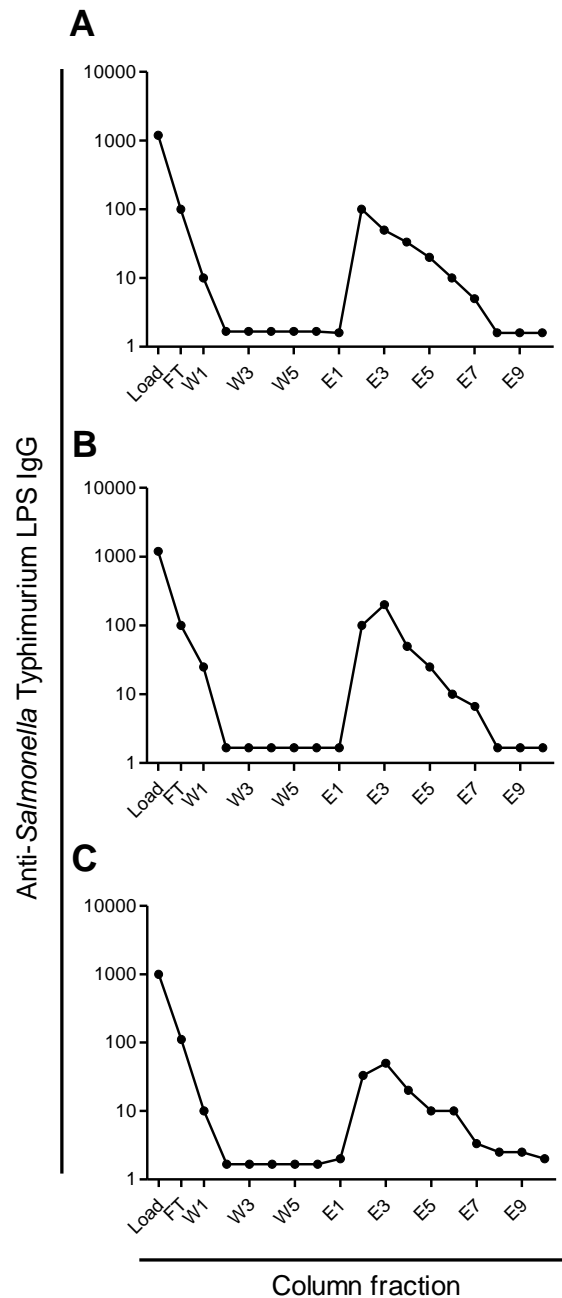


Figure 6.9 Variation in amount of activated OAg immobilised to NHS-Sepharose affects recovery of bound antibody from affinity columns.

300 μ l ammonium sulfate-treated human serum containing anti-*Salmonella* Typhimurium antibodies corresponding to 1200 ELISA units, was applied to three OAg-ADH columns each with varying concentration of OAg-ADH linked to 1 ml NHS-Sepharose: (A) 3.5 mg/ml, (B) 1 mg/ml, (C) 0.5 mg/ml and the flow through (FT) was collected. Each column was washed with 6 ml PBS (W1-3), 6 ml 50 mM NaPi, 500 mM NaCl pH 7.2 (W4-6) and bound antibody was eluted with 5 ml 0.1 M glycine, 0.1 M NaCl pH 2.4 (E1-10). Concentration of anti-OAg IgG antibody in each fraction was tested by ELISA using LPS coated plates. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Each panel represents data using one column and is representative of 3 experiments.

6.3.10 PURIFICATION OF ANTI-O-ANTIGEN ANTIBODIES FROM HIV-INFECTED MALAWIAN ADULT SERUM

The main aim of this project was to develop a method which could be used to affinity purify anti-OAg antibodies from various human serum to enable further characterisation of the role of these antibodies in cell-mediated immunity to NTS. All of the method development and optimisations were performed using serum from a healthy HIV-uninfected adult, which was known to contain antibodies to the LPS of *Salmonella* Typhimurium D23580 as this is in plentiful supply allowing the same sera to be used for method optimisation and comparison of modifications. The maximum yield we had obtained following all possible optimisations was 51% with this serum (Figure 6.9 B).

We confirmed that the developed method was applicable to other sera by applying a protein precipitation solution of serum taken from a HIV-infected African adult. Anti-*Salmonella* Typhimurium OAg IgA and IgM as well as IgG were removed from the serum, confirming that the produced columns are not isotype selective and are able to remove IgG, IgA and IgM antibodies specific to the OAg. The percentage of IgA eluted was 34% (Figure 6.10 B) and 26% for IgM (Figure 6.10 C). The recovery of IgG against the OAg was increased to 58% for this serum (Figure 6.10 A). This serum has a higher anti-LPS antibody titre compared to the HIV-uninfected serum which was used for all of the method developments, which may contribute to the higher yield of purified antibody.

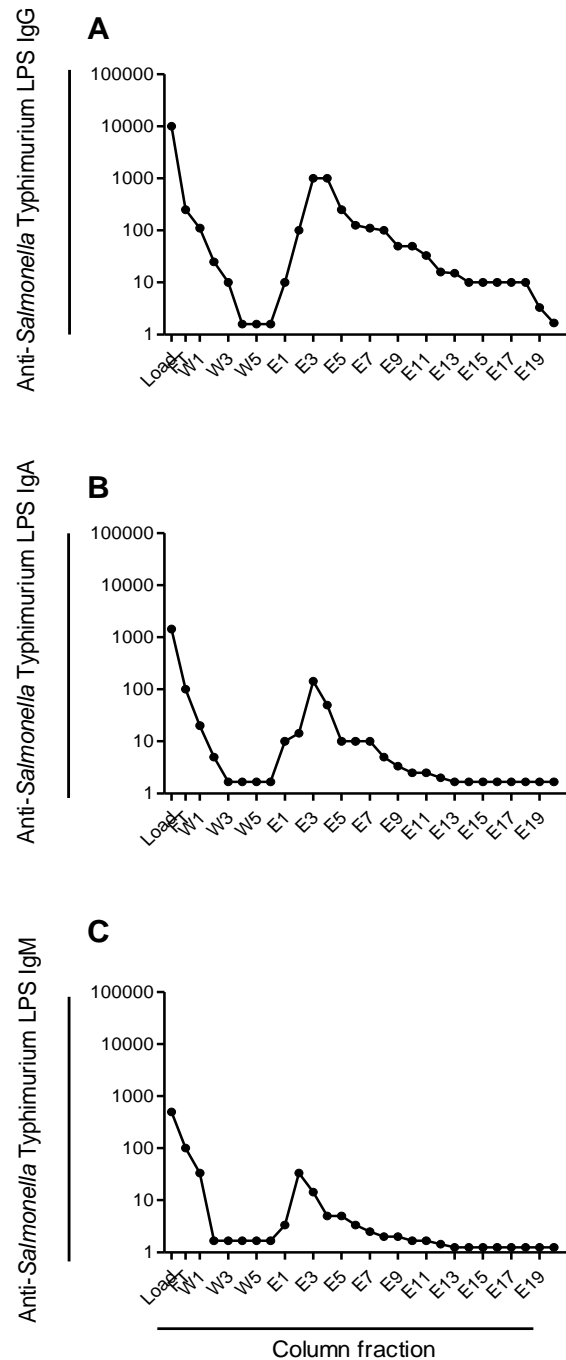


Figure 6.10 Purification of IgG, IgA and IgM antibodies to OAg of *S. Typhimurium* from a HIV-infected African adult serum.

ELISA profile for the elution of human anti-*S. Typhimurium* antibodies from serum from a HIV-infected African adult. 300 μ l of ammonium sulphate-precipitated human serum proteins with an anti-*Salmonella* Typhimurium antibody concentration corresponding to 10000 (IgG) ELISA units was applied to an OAgADH column (load). Flow through (FT) containing unbound antibody was collected, and the column washed with 6ml PBS (W1-3), followed by 6 ml 50 mM NaPi, 50 mM NaCl pH 7.2 (W4-6). Bound antibodies were eluted with 10 ml 0.1 M glycine, 0.1 M NaCl pH 2.4 in 500 μ l fractions (E1-20). All fractions were collected and the concentration of anti-LPS IgG (A), IgA (B) or IgM (C) antibody in each fraction was tested by ELISA for antibody to LPS. Reciprocal of concentration of antibody giving an OD of 1 at λ 405nm is plotted against column fraction collected. Each panel represents data using one column and is representative of 5 experiments.

6.4 DISCUSSION

One of the overall aims of this PhD is to study the role of antibody to *Salmonella* in cell-mediated immunity in the context of HIV-infected adults. High titres of antibodies against the OAg of *Salmonella* Typhimurium have been observed in a subset of HIV-infected adults in Malawi and these have been shown to inhibit killing by complement (61). To enable further characterisation of the potential opsonic role of these antibodies in cell-mediated immunity to NTS we needed to isolate them from human serum. Since commercial columns do not exist, we developed a new approach to affinity purify these antibodies.

We successfully coupled purified activated OAg from invasive African *S. Typhimurium* D23580, to NHS-Sepharose using ADH as a linker, to produce an affinity column. Insertion of hydrazide groups onto the OAg was carried out using two different procedures and resulted in the cross-linking of purified activated O-Ag to an N-hydroxysuccinamide matrix. In one case (OAgADH), one OAg chain was linked via the KDO group at the end of the core region to a single ADH molecule. This method does not alter the available epitopes along the saccharide chain. The other immobilised antigen, OAgoxADH, underwent prior oxidation resulting in multiple ADH molecules linked to the OAg chain, potentially enhancing the binding capacity of the OAg to the NHS-Sepharose but also modifying the OAg chain structure. Since the OAg remains attached to the oligosaccharide core, the antibodies which are purified from the human serum will contain antibodies targeting the core as well as antibodies specific for the OAg.

One of the main caveats when selecting and producing an affinity column is that modification of the antigenic structure often occurs during activation or coupling of the ligand to the matrix, and the affinity of an antigen for the corresponding antibody is frequently reduced (216). Both OAgADH and OAgoxADH columns bound more than 90% of the applied commercial anti-*Salmonella* O:4,5 rabbit antiserum and more than 75% of the polyclonal antibodies from human serum, suggesting that the acid hydrolysis used to purify OAg and activation with ADH had not altered the antigenic determinants present on the OAg chain. Binding of antibodies in the human serum to the resin could not be improved by using whole serum or precipitated human serum proteins which had passed through a Vivaspin column to remove small impurities. Both resins gave a similar high recovery of commercial rabbit anti-*Salmonella* O:4,5 antibodies. However, when human serum was applied to the resins, the recovery of antibodies was lower than that observed for the rabbit antiserum. The yield of human purified antibody was higher for the OAgADH column compared to the OAgoxADH column, and consequently this OAg activation method is preferred.

Human serum is polyclonal and contains many different antibodies which recognize and bind different epitopes on the same antigen with different binding affinities. Such antigen-antibody binding involves many weak interactions, including hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions (218-220). Therefore effective elution of polyclonal antibodies may require many different elution buffers (209;221). Glycine at acidic pH is commonly used to elute antibodies from antigen-affinity columns and for our columns, 0.1 M glycine, 0.1 M NaCl pH 2.4 was the most effective elution

buffer. Other elution buffers such as 20% ethanol to investigate the effect of an organic solvent, 100 mM Tris pH 9 as alkaline buffer, 8 M urea as a denaturant and 4 M MgCl₂ to raise the ionic strength of the solvent, with an accompanying weak chaotropic effect were tested in an attempt to increase the recovery, and MgCl₂ removed 18% of the bound antibody. When combined sequentially with glycine, MgCl₂ was unable to elute any remaining bound antibody suggesting that both buffers remove the same sub-population of antibody.

It is also possible that the majority of antibodies applied to the column could have been successfully removed following elution under acidic conditions, but that all of the eluted antibodies were not fully renatured and therefore no longer able to bind to LPS coated plates in the ELISA. However, Narhi *et al* confirmed that antibodies can refold to their native conformation following elution with glycine at a low pH provided they are neutralised and dialysed immediately (221). We did not investigate the effect of the elution buffers on their conformation and so although we neutralised and dialysed the eluted antibodies immediately, we cannot exclude than an irreversible denaturation occurred. We can only presume that the remainder of the antibodies which are not accounted for in the flow through, washes or eluates remain bound to the OAg-resin and could not be eluted using any of the conditions tested.

We verified that the ratio of antigen to antibody affected antibody elution. Reduction in the amount of OAgADH coupled to the resin from 3.5 mg to 1 mg per ml of resin, doubled the recovery of purified antibody and decreasing the concentration of linked

OAgADH further to 0.5 mg per ml of resin decreased the recovery, verifying that the ratio of antigen to antibody affected antibody elution. Differential loading of the antigen on the resin could result in different OAg chain conformations and a different exposure of the epitopes and/or determine a different interaction with the corresponding antibodies.

The present study describes the successful coupling of OAg from *S. Typhimurium* to NHS-Sepharose resin to produce an affinity matrix that is capable of purifying specific IgG, IgA and IgM antibodies from polyclonal human serum. The columns tested were used at least ten times, with no deleterious effect on the recovery of antibodies and it would be interesting to investigate further if this process could be increased to a large scale purification of antibodies against both the OAg from *Salmonella* and other bacterial polysaccharides. Since the antibodies which were removed from the serum could be antibody against the core region, as well as the OAg, the purified antibody could be absorbed with the core oligosaccharide to remove these antibodies and leave anti-OAg specific antibody in the eluates prior to use.

6.5 CONCLUSIONS

We developed a method to purify anti-*S. Typhimurium* D23580 antibodies from human serum using affinity chromatography. Purified *Salmonella* Typhimurium OAg, attached to the core oligosaccharide was activated with ADH via two different chemistries

before linking to NHS-Sepharose resin. One method modified the OAg chain (OAgoxADH) and the other method did not alter the chain (OAgADH). Both columns reversibly bound polyclonal rabbit antibodies, with high recovery of purified antibody. OAgADH was the preferred column for extractions from human serum as the OAg chain is not modified. Although 75% of antibodies in precipitated human serum which was applied to the column bound, the initial recovery of purified antibody was low. Different elution buffers were tested and 0.1 M glycine, 0.1 M NaCl pH 2.4 removed the most functional antibody. Using two buffers sequentially did not increase the recovery of antibody. Optimal yields were obtained by decreasing the amount of activated OAg per ml of resin from 3.5 mg to 1 mg OAgADH. The highest recovery of anti-*S. Typhimurium* OAg-core antibody from a HIV-uninfected serum was 51% and 58% for HIV-infected adult serum which has a higher anti-LPS titre. The purified antibodies can be used to further study the role of anti-OAg antibodies in immunity to NTS.

CHAPTER 7.

OPSONIC ANTIBODY AND IMMUNITY TO NTS IN MALAWIAN CHILDREN WITH BACTERAEMIA

7.1 INTRODUCTION

NTS are a major cause of invasive bacterial disease in African children (18-20). In Malawi, NTS are the commonest cause of bacteraemia (21), with case fatality rates of 24% in children (28;199). NTS bacteraemia is associated with malnutrition, young age, HIV infection and malaria (18;20;21) in African children. NTS bacteraemia mainly occurs in children aged 4-24 months (20;49). Few cases of NTS bacteraemia were observed in children less than 4 months, suggesting that maternal antibody protects during this phase and that as levels of maternal antibody decrease, children are increasingly susceptible to bacterial infection (50). It has previously been shown that a lack of antibody in healthy young Malawian children's sera corresponded with absent bactericidal killing (50) and reduced killing by peripheral blood cells (151) .

In order to develop a vaccine for NTS for use in Africa, an improved understanding of the acquisition of antibody in response to NTS infection and the protective efficacy of such antibody is required. In this chapter we investigated peripheral blood cell killing of an African isolate of *Salmonella* after opsonisation with antibody from Malawian children with NTS bacteraemia.

7.2 OBJECTIVES

The aim of this work was to study opsonic antibody to NTS in Malawian children with NTS bacteraemia. Using serum from Malawian children infected with NTS and a follow-up sample four weeks post infection, we investigated whether antibody in these children can facilitate phagocytic cell-mediated killing of NTS.

7.3 RESULTS

7.3.1 STUDY PARTICIPANTS

Sera were available for 156 children aged 0 to 14 years who presented to QECH, Blantyre, Malawi with NTS bacteraemia between 1st January 2006 and 31st December 2006 and whom had been recruited into a prospective study of NTS bacteraemia in African children. Invasive *Salmonella* infection was confirmed by blood culture. Blood was venesected from the children upon recruitment and serum prepared from the clotted blood and stored at -80°C (acute samples). Serum samples were also prepared from children in convalescence 4 weeks after the onset of *Salmonella* infection (convalescent samples), and stored at -80°C. 119 acute and 119 convalescent samples were available for assays to assess the opsonic activity of antibody in cell-mediated killing assays and anti-*Salmonella* antibody level analysis, and for 82 children paired acute and convalescent samples were available.

7.3.2 *SALMONELLAE* OPSONISED WITH ACUTE AND CONVALESCENT SERA FROM SOME CHILDREN COULD NOT BE KILLED BY PERIPHERAL WASHED ADULT BLOOD CELLS

We first investigated if *S. Typhimurium* D23580, a representative invasive Malawian isolate of NTS, opsonised with serum from children with NTS bacteraemia (acute sera) and sera taken 4 weeks after admission (convalescent sera) could be killed by blood cells. Washed peripheral blood cells from a healthy adult donor were inoculated with opsonised *Salmonellae* at a concentration of 1×10^5 cfu/ml at 37°C, and viable *Salmonellae* numbers were determined after 45, 90 and 180 min.

Salmonellae opsonised with 76% (90/119) of the acute sera underwent at least a 1 log₁₀ kill after 180 min (median kill -2.0, range 1.7 to -3.0) (Figure 7.1), which is designated as the 'normal kill' in this assay when using sera from healthy HIV-uninfected adults which contains antibody and complement. Bacterial growth or a kill of less than 1 log₁₀ was observed with 24% (29/119) of the acute sera tested. Opsonisation of *Salmonellae* with 61% (73/119) of the convalescent sera resulted in a 1 log₁₀ kill or greater by washed peripheral blood cells, with killing of *Salmonella* absent or impaired when opsonised with 39% (46/119) of the convalescent sera (Figure 7.1). Blood cell killing of *Salmonellae* opsonised with convalescent sera (median kill -1.1, range 1.9 to -3) was significantly reduced compared to acute sera (median kill -2.0, range 1.7 to -3, Mann-Whitney U test p=0.0002). The acute sera were from children presenting to QECH with NTS bacteraemia, almost all of whom had commenced antibiotic treatment prior to venesection, and so the increased kill observed when opsonising with acute sera may be

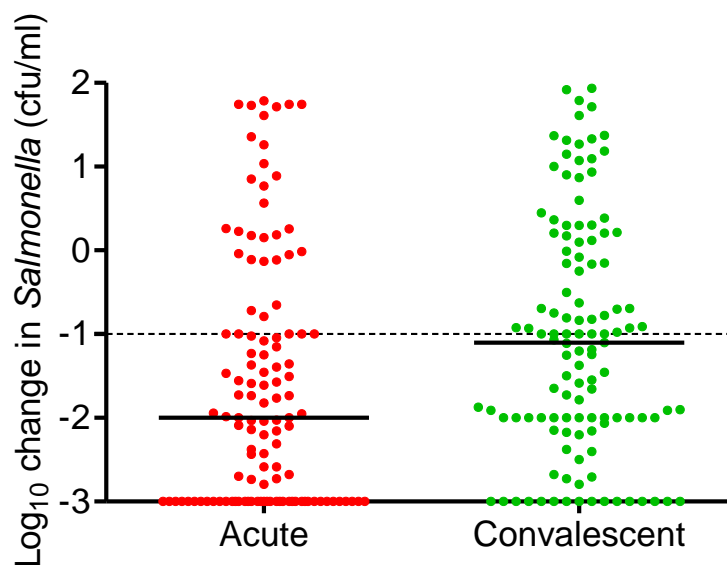


Figure 7.1 Blood cell killing of *S. Typhimurium* D23580 opsonised with acute and convalescent sera.

In vitro killing of *S. Typhimurium* D23580 opsonised with 119 acute sera from Malawian children with NTS bacteraemia, and 119 convalescent sera from children 4 weeks post-infection. Blood cell killing of convalescent sera was significantly reduced compared to killing of acute sera (MW test $p=0.0002$). Initial concentration of *Salmonellae* is 1×10^5 cfu/ml, with log change in *Salmonellae* concentration after 180 min incubation with washed adult peripheral blood cells shown. Negative values show a decrease in viable *Salmonellae* and positive values show an increase in viable *Salmonellae* compared with the initial starting concentration. Each circle represents killing of *Salmonella* opsonised with serum from one child. The dotted horizontal line indicates normal blood cell killing of *Salmonella* opsonised with healthy adult Malawian serum and the solid black horizontal lines indicate median kill.

due to the antibiotics killing the bacteria. More than 90% of the children were taking gentamicin, ciprofloxacin or ceftriaxone at the time that their acute blood sample was taken. *S. Typhimurium* D23580 is sensitive to gentamicin, ciprofloxacin, ceftriaxone and is resistant to ampicillin, chloramphenicol and cotrimoxazole.

7.3.3 EFFECT OF AGE ON CELL-MEDIATED IMMUNITY TO NTS IN BACTERAEMIC CHILDREN

We next investigated if an immature immune system could explain why some sera from children with acute NTS bacteraemia and children in convalescence could not kill *S. Typhimurium* D23580. Similar to previous studies of NTS bacteraemia in children, of the 119 children recruited to QECH over a one year period with acute NTS bacteraemia, 77% were under 36 months, and the median age was 16 months. Only 8 acute sera and 9 convalescent sera were from children aged less than 4 months old, which is consistent with the observation of MacLennan *et al* (50) that maternal antibody protects from birth to 4 months of age and that once maternal antibody titres wane, a peak in cases of NTS bacteraemia is observed.

Although there was no correlation between age and killing of *Salmonellae* opsonised with acute (Spearman $r_s=0.06$, $p=0.49$, 95% CI -0.12 to 0.24) (Figure 7.2 A) or convalescent sera (Spearman $r_s=-0.12$, $p=0.17$, 95% CI -0.30 to 0.06) (Figure 7.2 B) after 180 min by washed blood cells, 59% (17/29) of the acute sera and 70% (32/46) of the

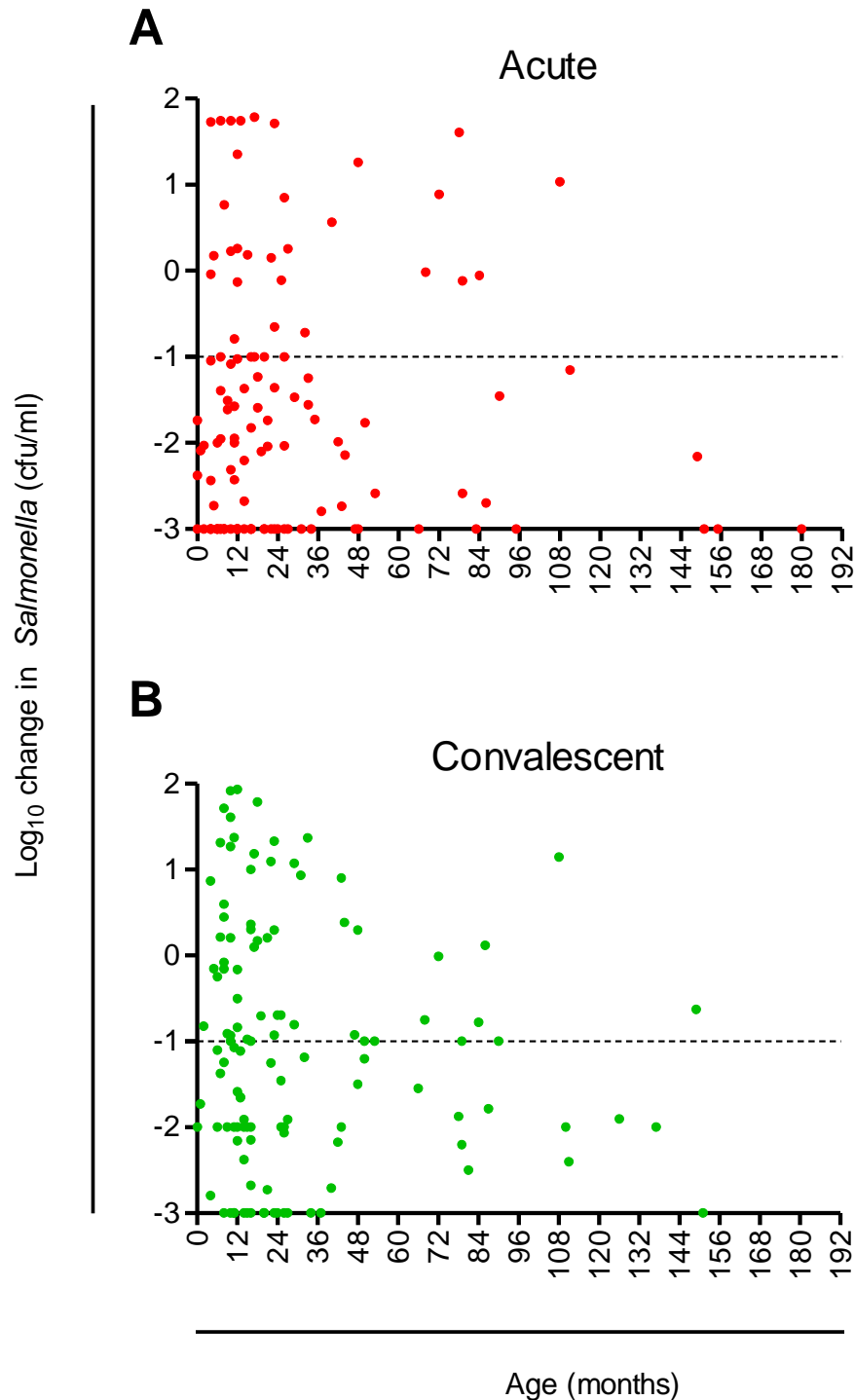


Figure 7.2 No correlation between age and impaired killing of *Salmonella* opsonised with acute and convalescent sera.

There was no correlation between killing of *Salmonellae* opsonised with acute sera (A) ($r_s = 0.06$, 95% CI -0.12 to 0.24, $p=0.49$) or convalescent sera (B) ($r_s = -0.12$, 95% CI -0.3 to 0.06, $p=0.17$) by washed peripheral blood cells after 180 min and the age of the child from whom the serum was taken. Each circle represents killing of *Salmonellae* opsonised with serum from one child. The dotted horizontal line indicates normal blood cell killing of *Salmonellae* opsonised with healthy adult Malawian serum.

convalescent sera which were unable to induce cell-mediated killing of bacteria were from children under 24 months old. At this age, the children do not have a fully mature immune system which could account for why some of the acute and convalescent sera cannot kill *Salmonella*.

7.3.4 HIV INFECTION AND BACTERAEMIC CHILDREN

HIV infection is known to be associated with NTS bacteraemia in African children and adults (30). We assessed whether a lack of opsonic activity of some acute and convalescent sera was associated with HIV-co-infection. 47 of the acute sera were from children who were HIV-infected, but 75% (35/47) of these sera were able to induce a 1.0 log₁₀ kill of *S. Typhimurium* D23580. For the convalescent sera, 38 were from HIV-infected children, with 50% (19/38) of these sera able to kill *S. Typhimurium* D23580. 46% (12/26) of the acute sera and 41% (19/46) of the convalescent sera which were unable to facilitate blood cell killing of *S. Typhimurium* D23580 were from HIV-infected children. The median kill by sera from HIV-infected acute bacteraemic children was not significantly different compared to the median kill by sera from HIV-uninfected acute sera (median kill for acute HIV+ve -1.7 versus HIV-ve -2, Mann Whitney test p=0.48) (Figure 7.3 A). Again, the presence of antibiotics in the serum makes interpretation of this result difficult. However, for the convalescent sera, the median kill for HIV-infected serum was significantly lower than that for HIV-uninfected serum (-0.8 versus -1.4, Mann Whitney test p=0.03) (Figure 7.3 B).

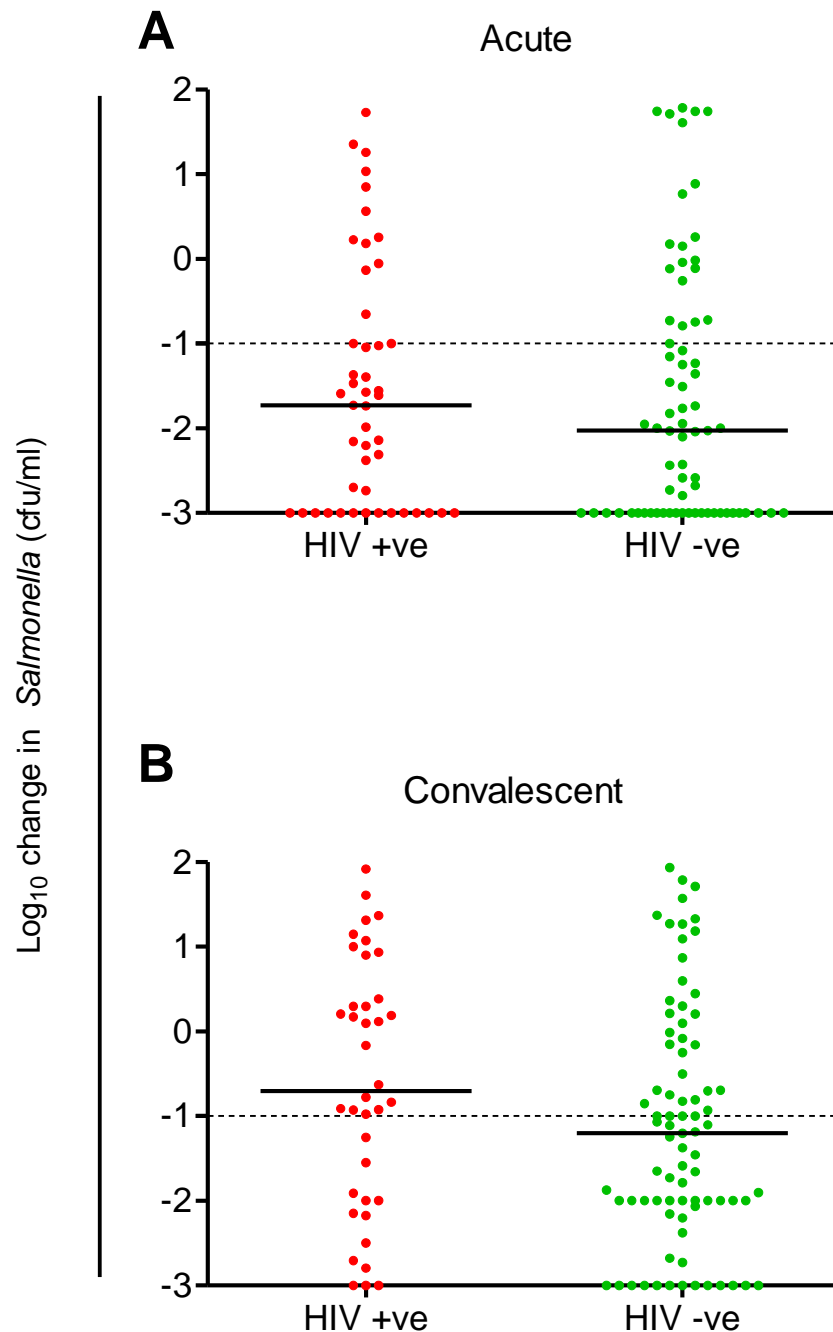


Figure 7.3 Blood cell killing of *S. Typhimurium* D23580 opsonised with HIV-infected acute and convalescent sera from African children with NTS bacteraemia. (A) Blood cell killing of *Salmonellae* opsonised with HIV-infected (HIV +ve) or HIV-uninfected (HIV -ve) acute sera or, (B) HIV-infected/un-infected convalescent sera from Malawian children. Initial concentration of *Salmonellae* is 1×10^5 cfu/ml with change in viable *Salmonellae* shown after 180 min incubation with washed adult peripheral blood cells. Negative results show a decrease in viable *Salmonellae*. Each circle represents killing of *Salmonellae* opsonised with serum from one child. The dotted horizontal line indicates normal blood cell killing of *Salmonellae* opsonised with healthy adult Malawian serum and the solid black horizontal lines indicate median kill.

7.3.5 ANTI-SALMONELLAE ANTIBODY TITRES AND BLOOD-CELL KILLING OF SALMONELLAE OPSONISED WITH ACUTE AND CONVALESCENT CHILDREN'S SERA

Antibody and complement are required for optimal cell killing of *Salmonella* (151), and therefore, the observed absence of killing of *Salmonella* opsonised with 24% of the acute and 39% of the convalescent sera could be the result of a lack of antibody or complement. We presume that complement remains functional in all of the sera, as sera were optimally prepared and frozen at -80°C since separating from clotted blood. However, deposition of C3 by these sera on the surface of *Salmonella* Typhimurium D23580 was not tested, so we cannot confirm lack of complement deposition, or increased complement consumption, which can occur in sepsis, as an explanation for a lack of *Salmonella* killing by some of the acute and convalescent sera.

To investigate if an absence of antibody against *S. Typhimurium* D23580 in some of these sera could explain the reduced killing, the binding of IgG, IgA and IgM to *S. Typhimurium* D23580 were quantified using flow cytometry. All antibody assays in this chapter were performed by Dr Esther Gondwe, in work prior to this PhD as part of a larger study on the role of bactericidal antibody in immunity to NTS in children with bacteraemia. The protective anti-*Salmonella* titres in the assay were set at 1.5 U, which has previously been found to be the threshold required for antibody-mediated killing of NTS (50). 84% (100/119) of the acute sera had anti-*Salmonella* IgG levels exceeding 1.5 U, but 23/100 of these acute sera were not able to kill *Salmonellae* (Figure 7.4 A). 16% (19/119) of the acute sera had IgG titres less than 1.5 U, but *Salmonellae* opsonised with

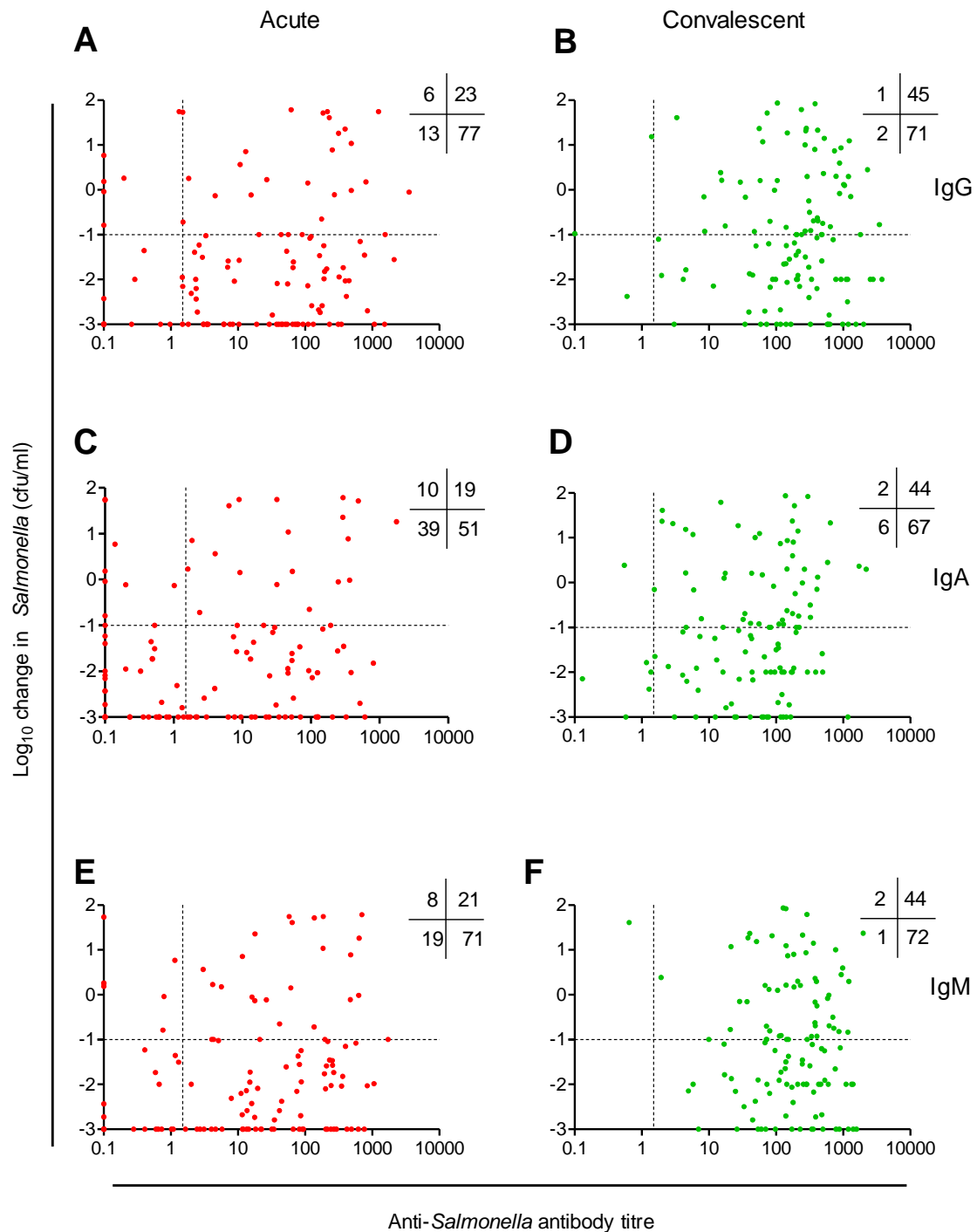


Figure 7.4 Blood cell killing of *S. Typhimurium* D23580 and anti-*Salmonella* IgG, IgA and IgM levels in sera from Malawian children.

In vitro killing of *S. Typhimurium* D23580 opsonised with serum from acute and convalescent sera by adult washed blood cells after 180 min compared with anti-*Salmonella* IgG (A, B), IgA (C, D) and IgM (E, F) levels in the serum used to opsonise the bacteria. Each circle corresponds to serum from one child. Dotted vertical line corresponds to 1.5 U of antibody which is the threshold required to induce antibody-mediated killing of bacteria and the dotted horizontal line indicates threshold of blood cell killing of *Salmonellae* opsonised with healthy adult Malawian serum.

13/19 of these sera were killed by peripheral blood cells. Killing of *Salmonellae* in the absence of sufficient IgG could be antibiotic-mediated. *Salmonellae* opsonised with 6 sera which had less than 1.5 U IgG, also had less than 1.5 U of IgA and IgM and were unable to kill *Salmonella*, suggesting a lack of total anti-*Salmonella* antibody in these sera.

Anti-*Salmonella* IgG levels less than 1.5 U were present only in 3 (2.5%) of the 119 convalescent sera, but opsonisation with 2 of these sera resulted in killing of *Salmonellae* by peripheral blood cells (Figure 7.4 B). IgG levels exceeding 1.5 U were detected in 97.5% (116/119) of the convalescent sera tested, but *Salmonellae* opsonised with 45 of these sera were not killed by peripheral blood cells. There was a weak correlation between anti-*Salmonella* IgG titre for the acute sera ($r_s = 0.17$, $p = 0.05$, 95% CI -0.01 to 0.34) but no correlation for the convalescent sera ($r_s = 0.01$, $p = 0.86$, 95% CI -0.16 to 0.20) and killing by washed peripheral blood cells.

59% (70/119) of the acute children's sera had IgA levels above the 1.5 U protective threshold. 19/70 of these failed to induce blood cell killing of *Salmonella* (Figure 7.4 C). 41% (49/119) of the acute sera had anti-*Salmonella* IgA levels less than 1.5 U. *Salmonellae* opsonised with 39/49 of these sera were killed by peripheral blood cells which could be the result of antibiotic-mediated killing or IgG inducing cell-mediated killing of the bacteria. 6 of the 10 sera which had less than 1.5 U of IgA, also had less than 1.5 U of IgG anti-*Salmonella* antibody which is likely to explain why these sera

were not able to induce blood cell killing of *S. Typhimurium* D23580. For the convalescent sera (Figure 7.4 D), only 8 (7%) sera had IgA levels less than 1.5 U. All 8 of these sera had >1.5 U of IgG and IgM so it is not clear why 2 of these sera were not able to kill *Salmonella*. No correlation between *S. Typhimurium* IgA level and killing by blood cells was observed for the groups of acute ($r_s = 0.06$, $p=0.37$, 95% CI -0.12 – 0.24) or convalescent ($r_s = 0.14$, $p=0.58$, 95% CI -0.23-0.13) sera. This is to be expected as IgA is not opsonic and consequently the presence of anti-*Salmonella* IgA antibody is unlikely to have an effect on cell-mediated killing of the bacteria.

23% (27/119) of the acute sera had anti-*Salmonella* IgM titres less than 1.5 U, with blood cell-killing absent for 8 of these sera (Figure 7.4 E). 6 of these sera also had less than 1.5 U of IgG and the other 2 sera had borderline levels of IgG (1.84 U) which could explain the lack of blood cell-killing by these sera. Killing was also absent for 21 sera which had anti-*Salmonella* IgM titres more than 1.5 U. Only 3 convalescent sera had anti-*Salmonella* IgM titres below 1.5 U (Figure 7.4 F), and 2 of these 3 sera failed to induce blood cell killing of *Salmonella*, despite the presence of IgG. As for IgA, there was no correlation between anti-IgM levels and killing of *Salmonellae* opsonised with acute ($r_s = 0.09$, $p=0.28$, 95% CI -0.08 to 0.27) or convalescent ($r_s = -0.05$, $p=0.58$, 95% CI -0.23 to 0.13) sera by washed blood cells. This is to be expected as IgM cannot directly act as an opsonin itself since human phagocytes lack an FcR for IgM, but IgM can have an indirect opsonic effect through its enhanced ability to fix complement.

7.3.6 ANTI-SALMONELLA ANTIBODY LEVELS INCREASE AFTER INFECTION

The previous result suggested that anti-*Salmonella* antibody levels were increased for the convalescent phase sera compared to the acute phase sera. To confirm this, anti-*Salmonella* IgG, IgA and IgM levels in the 82 paired sera which were available from Malawian children both at presentation with acute NTS bacteraemia and at convalescence were compared (Figure 7.5). The median anti-*Salmonella* IgG level for the 82 acute sera was four times lower than the median level in convalescence (53.69 U compared to 230.8 U, Mann-Whitney test $p < 0.0001$). The median anti-*Salmonella* IgA levels were also significantly increased for the convalescent sera compared to the acute sera (median 43.49 U compared to 7.06 U, Mann-Whitney test $p < 0.0002$). The same was found for anti-*Salmonella* IgM levels, with the median IgM level rising from 4.52 U for the acute sera to 151.2 U for the convalescent sera (Mann-Whitney test $p < 0.0001$). These observations confirmed that, as expected, anti-*Salmonella* antibody levels increase after infection with Malawian isolates of NTS.

7.3.7 BLOOD CELL KILLING OF SALMONELLA IS ENHANCED WHEN OPSONISED WITH DILUTIONS OF ACUTE AND CONVALESCENT SERA

Following the previous observation that some sera were unable to induce cell-mediated killing of *Salmonella* despite the presence of high levels of anti-*Salmonella* antibodies, we investigated the effect of diluting antibody within the acute and convalescent sera on opsonisation and induction of cell-mediated clearance of the bacteria from the blood.

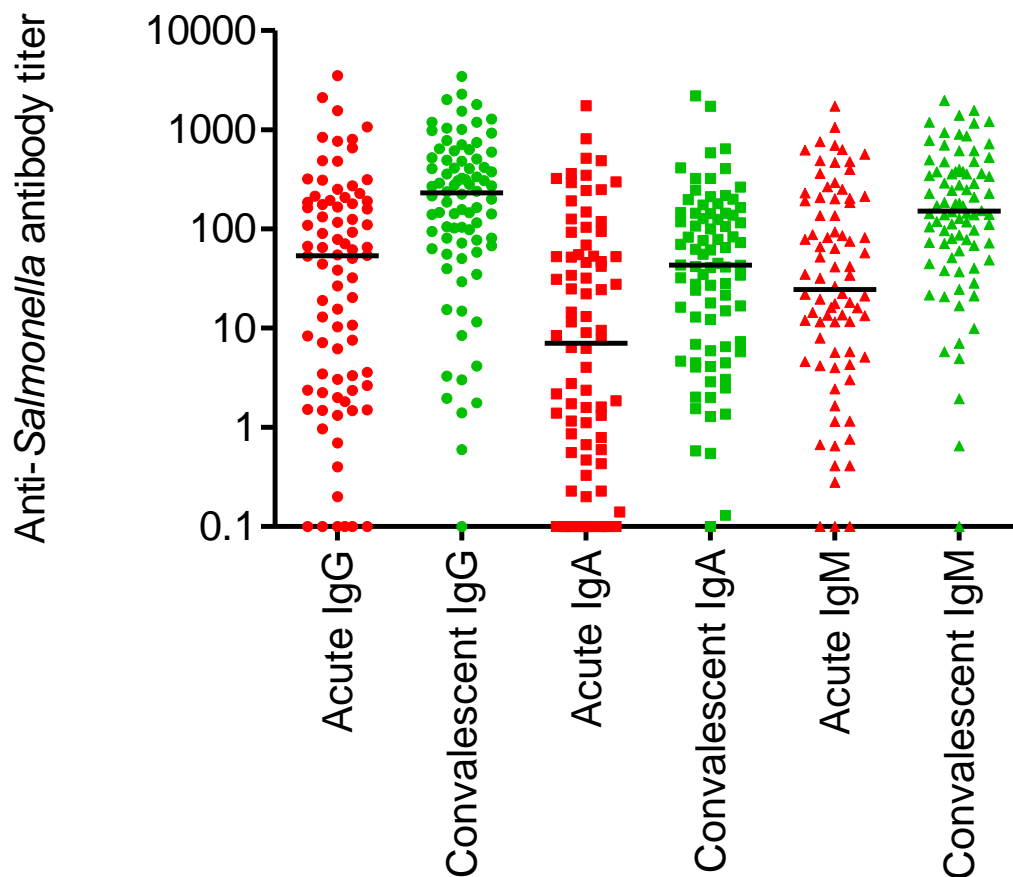


Figure 7.5 Anti-*Salmonella* antibody levels are elevated in convalescent sera compared to acute sera in children with NTS bacteraemia.

Anti-*Salmonella* Typhimurium D23580 IgG, IgA and IgM levels in serum from 82 Malawian children with NTS bacteraemia (acute) and 4 weeks after infection (convalescent) as assessed by flow cytometry, with median titre indicated with a black line.

Using a method we had previously optimised (see Chapter 4), *Salmonellae* were opsonised with 40% acute or convalescent sera, which was heat-inactivated to destroy endogenous complement and diluted 5 fold with PBS (dilution range 1:5 – 1:78,125) and 60% antibody-depleted fresh human serum as a complement source. 6 acute (Figure 7.6 A) and 6 convalescent sera (Figure 7.6 B) were first tested. When opsonised with undiluted serum, only 1 of the 6 acute sera induced over a 1 log₁₀ kill of *S. Typhimurium*, but when the sera were diluted 1:5 and supplemented with complement, all six effected at least a 1 log₁₀ kill of *Salmonellae* by washed blood cells (Figure 7.6 A). When diluted to 1:25, killing was diminished for 2 sera. At a serum dilution of 1:125, kill was lost for 3 further sera with only one serum able to kill at 1:625, but this kill was lost at a serum dilution of 1:3125. For the 6 convalescent sera (Figure 7.6 B), only 2 of the sera when used undiluted effectively opsonised *Salmonellae* and induced a 1 log₁₀ kill or greater by washed blood cells. *Salmonellae* underwent at least a 1 log₁₀ kill by blood cells when opsonised with complement and heat-inactivated sera diluted to 1:5 and 1:25. At a dilution of 1:125, only 4 sera could sustain *Salmonella* killing, with 3 sera unable to kill at 1:625, with killing lost for the final serum at a dilution of 1:3125.

These results indicate that blood cell killing can be enhanced when antibody is diluted. Those sera which were unable to induce cell-mediated killing when used undiluted as opsonins were able to kill when diluted. To determine if killing could be enhanced for all sera, all 119 acute (Figure 7.6 C) and 119 convalescent sera (Figure 7.6 D) were diluted and tested in the cell killing assay. 39% (35/90) of the acute sera that could

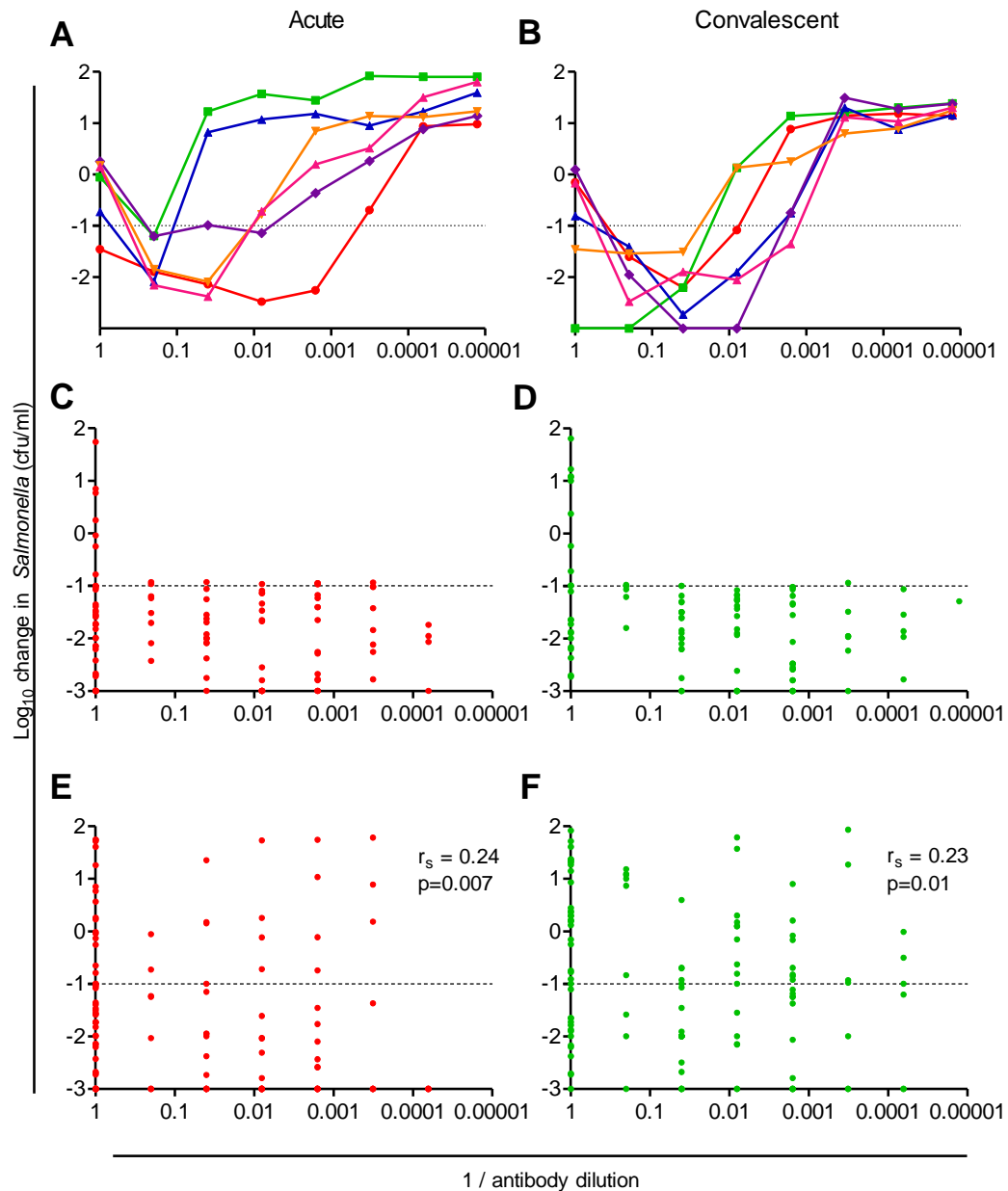


Figure 7.6 Blood cell killing of *Salmonella* opsonised with diluted children's sera.

Killing of *S. Typhimurium* D23580 opsonised with 60% antibody-depleted serum and with 40% diluted (1:5, 1:25, 1:125, 1:625, 1:3125, 1:15625) heat-inactivated acute (A) or convalescent (B) children's serum as an antibody source. Log₁₀ change in *Salmonellae* after 180 min plotted against 1/antibody dilution, with negative results showing killing of *Salmonellae* and positive results representing bacterial growth. Log₁₀ change in *Salmonellae* observed at the maximum dilution of (C) acute or (D) convalescent heat-inactivated serum which induces at least a 1 log₁₀ kill of *Salmonella* by peripheral washed blood cells. Sera on the y axis show sera which could not kill when diluted and kill/growth when used undiluted as an opsonin. Log₁₀ change in *Salmonellae* induced by blood cells with *Salmonellae* opsonised with whole (E) acute or (F) convalescent serum plotted against the maximum dilution of the heat-inactivated serum which induces a 1 log₁₀ kill of *Salmonella*. Each circle represents data for serum from one child.

induce blood cell killing of *Salmonella* when whole serum was used to opsonise the bacteria, could not kill when diluted, even at dilutions as small as 1:5. 59% (17/29) of the acute sera which were unable to induce a kill when used whole as an opsonin could kill when diluted, but 12/119 of the sera could not kill when used whole or diluted. 6 of these sera had lacked IgG, IgA and IgM titres in the whole serum, so the inability to kill when diluted and supplemented with exogenous complement is likely due to the absence of anti-*Salmonella* antibody.

83% (61/73) of the convalescent sera which could effect a 1 log₁₀ blood cell kill of *Salmonella* when whole serum was used as an opsonin, could also kill when diluted (Figure 7.6 D). Absence of killing for the other 12 sera when diluted, again was likely to be due to insufficient antibody titres. For the 46 convalescent sera which were unable to facilitate killing when used undiluted, 26 were able to effect a 1 log₁₀ kill when diluted, but 20 sera were unable to kill when used as a whole serum opsonin or when diluted and supplemented with human complement.

The serum dilution range over which antibody could be diluted and still effectively opsonise bacteria was similar for both groups of sera, with killing of *Salmonellae* observed at serum dilutions of 1:5 to 1:15625 (Figure 7.6 C and D). One convalescent serum could still kill following dilution to 1:78,125. The highest dilution at which the sera could kill *Salmonella* correlated with whole serum killing for the acute (Figure 7.6

E) (Spearman $r_s = 0.24$, $p = 0.0070$ 95% CI 0.06 to 0.41) and convalescent sera (Figure 7.6 F) (Spearman $r_s = 0.23$ $p = 0.01$ 95% CI 0.05 to 0.40).

7.3.8 KILLING OF *SALMONELLA* OPSONISED WITH DILUTED ANTIBODY BY PERIPHERAL BLOOD CELLS COMPARED WITH ANTI-*SALMONELLA* ANTIBODY LEVELS

We next investigated if the serum anti-*Salmonella* antibody levels correlated with the degree to which the sera could be diluted and still effect a killing of *Salmonella*. There was no correlation between anti-*Salmonella* IgG levels and the final dilution of serum which could still effect a $-1 \log_{10}$ kill when used to opsonise *S. Typhimurium* D23580 for the acute ($r_s = -0.14$, $p = 0.12$ 95% CI -0.31 to 0.04) (Figure 7.7 A) or convalescent sera ($r_s = -0.03$, $p = 0.67$, 95% CI -0.22 to 0.14) (Figure 7.7 B). There was a negative correlation between the anti-*Salmonella* levels and the final dilution of sera which could still effect a $-1 \log_{10}$ kill when used as an opsonin for the acute IgA ($r_s = -0.22$, $p = 0.02$, 95% CI -0.38 to 0.02) (Figure 7.7 C), acute IgM ($r_s = -0.24$, $p = 0.006$, 95% CI -0.41 to -0.06) (Figure 7.7 E) and convalescent IgM ($r_s = -0.2$, $p = 0.02$, 95% CI -0.37 to -0.01) (Figure 7.7 F). No correlation was observed between convalescent IgA antibody titres and final dilution of sera ($r_s = 0.04$, $p = 0.65$, 95% CI -0.14 to 0.22) (Figure 7.7 D).

7.4 DISCUSSION

NTS bacteraemia is a common cause of invasive disease in Africa. In this chapter we have reported 156 cases of paediatric NTS bacteraemia at QECH, Malawi in a 1 year

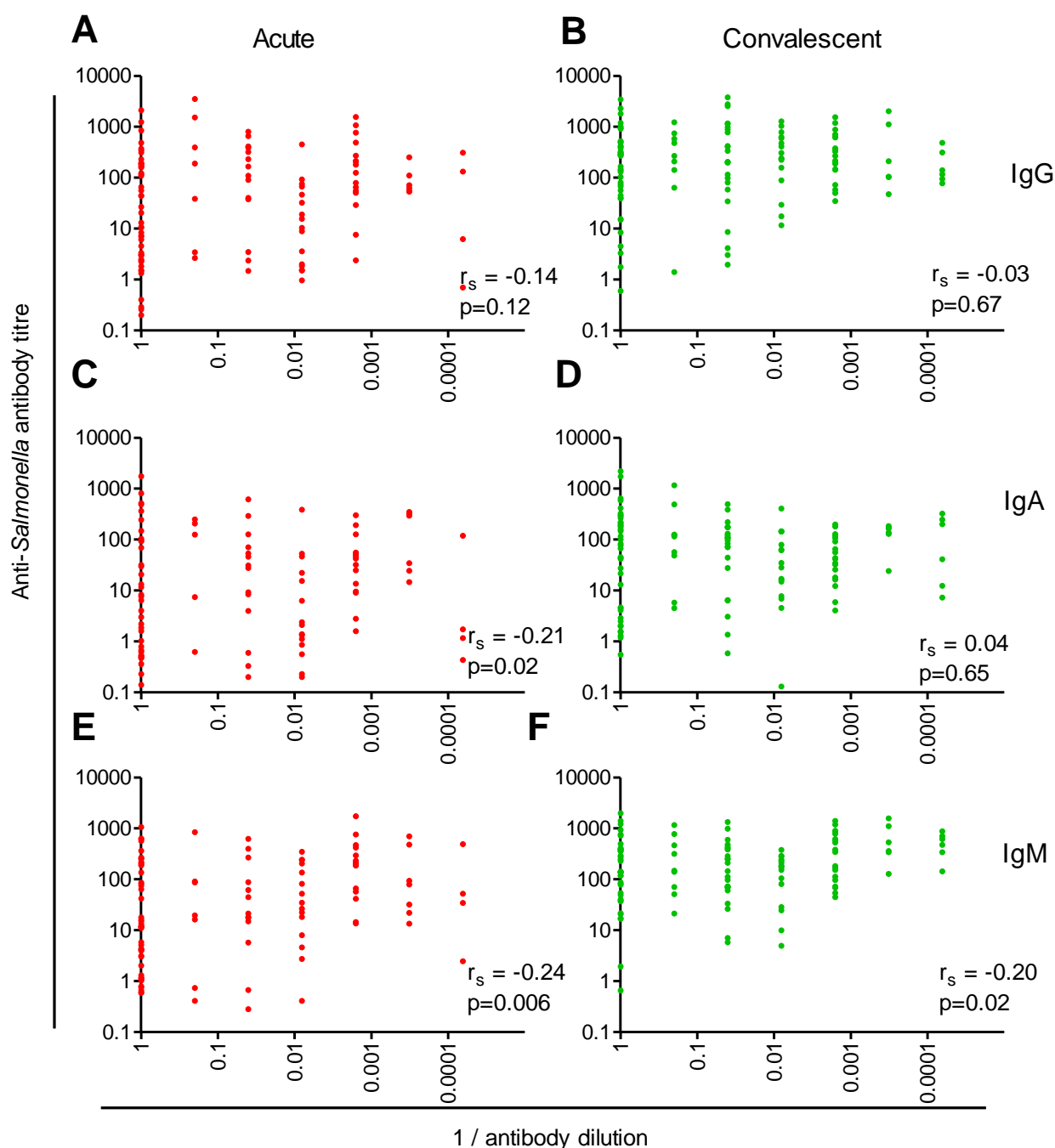


Figure 7.7 Anti-*Salmonella* antibody titre and maximum dilutions of sera able to effect *Salmonella* killing.

Anti-*Salmonella* IgG (A and B), IgA (C and D), IgM (E and F) levels in each of the acute (A, C, E) and convalescent (B, D, F) children's sera plotted against final dilutions of heat-inactivated serum which when supplemented with a human complement source could effectively opsonise *Salmonella* and induce a 1 log₁₀ kill of *Salmonella* by washed adult peripheral blood cells. Each circle represents data for serum from one child.

period from 1st January 2006 to 31st December 2006 highlighting the frequency of NTS infection. In keeping with previous NTS studies in Malawi (18;50) 84% of the children presenting to hospital with NTS bacteraemia were under 3 years of age. Although age did not correlate with ability to induce cell-mediated killing of NTS in the cohort of children studied in this chapter, 59% of sera from children at acute presentation and 70% of children in convalescence which were unable to kill *Salmonella* were under 24 months. At this age, children have not reached full maturity of their immune system and may be unable to initiate a rapid successful immune response against invading bacteria.

The availability of serum from Malawian children with acute NTS bacteraemia and at convalescence a month post-infection, provides a useful resource to study the acquisition of antibody during infection and how such antibody can kill *Salmonella*, as previous studies on immunity to NTS in African children have used sera from healthy children (50;151). The presence of antibiotics in the acute sera makes it difficult to ascertain the effect of blood-cell killing of *Salmonella* opsonised with acute sera. The increased kill effected by opsonisation with the acute sera is likely to be attributable to the effect of the antibiotics. Sera taken from children with NTS bacteraemia before administration of antibiotics would therefore be more informative. However, killing of *Salmonella* opsonised with acute sera could also be antibody-driven, as although it might be expected that children at acute presentation have no anti-*Salmonella* antibody, hence the reason they have NTS bacteraemia, they may actually have antibody as a

result of acute NTS infections, as the time of recruitment to the study follows an incubation and symptomatic period of infection prior to arrival at the hospital.

The children were infected with a range of invasive NTS isolates and not *S. Typhimurium* D23580, and this may explain why some children's sera were unable to kill *S. Typhimurium* D23580, because if the children were infected with *S. Enteritidis* and produce antibody to their infecting strain, then this may not be protective against *S. Typhimurium*, in particular *S. Typhimurium* D23580. However, the majority of sera were able to effectively opsonise and activate blood cell killing of *S. Typhimurium* D23580, suggesting that in some cases the antibodies produced during infection with different strains of *Salmonella* are cross-protective targeting common antigens.

It has previously been shown that serum from HIV-uninfected Malawian adults which contain anti-*Salmonella* antibody titres >1.5 U, facilitate a $2.0 \log_{10}$ kill of *Salmonella* by washed blood cells (151). All except 3 convalescent sera had >1.5 U IgG against *S. Typhimurium* D23580 post-infection. However, the presence of antibody did not necessarily correlate with protection against NTS infection, as 39% of the convalescent sera were unable to activate cell-mediated killing of *Salmonella* when whole sera were used for opsonisation. The impaired ability of these children to mount an appropriate antibody response to their infection could be the result of young age, an immature immune system or co-morbidities such as HIV, malaria and malnutrition. This observation could also be a result of the minimal antibody threshold used in the analysis. The flow cytometry assay used to quantify serum antibody titres may not be

sufficiently sensitive to quantify the threshold antibody titre needed to effect killing, and could give a saturated signal if there are large amounts of antibody binding to the bacteria. Furthermore, a lack of killing for some of the acute and convalescent sera could be due to impaired C3 deposition on the bacteria. This, however, is unlikely as the sera were optimally prepared and stored, but was not tested.

The observation that blood cell killing of *Salmonellae* opsonised with diluted convalescent sera was enhanced compared to whole serum for some children, could be an example of the Neisser-Wechsberg prozone phenomenon, where an excess of antibody inhibits killing, but as antibody is diluted killing is restored (222). For cell-mediated killing of *Salmonella*, excess total anti-*Salmonella* antibody could block or divert complement deposition or, alternatively, complement could be limiting in our assay, and when the sera are diluted and supplemented with exogenous complement (i.e. complement is no longer limiting), cell-mediated killing of *Salmonella* is enhanced. Our observation that killing is enhanced in some of these bacteraemic sera when serum is diluted suggests that there is something inhibiting the killing and when sera are diluted, killing is enhanced. We have previously observed that in some HIV-infected Malawian adults, high anti-OAg antibody titres inhibit cell-mediated killing of NTS, but at lower titres, these antibodies can be opsonic (see Chapter 5). Therefore, the high-antibody titres measured in the sera could be directed against the OAg of *Salmonella* and could be inhibiting the killing, but when diluted, the titre of these OAg antibodies decreases to a level at which they can be opsonic, or at lower titres complement deposition and access of antibodies to other targets such as those on the bacterial

membrane can occur, which could enhance killing by blood cells. Since the flow cytometry assay we use to quantify anti-*Salmonella* antibody titres measures antibody binding to a range of antigens on the whole bacteria, we do not know what proportion of the elevated anti-*Salmonella* antibody titres target the OAg. Performing ELISAs to quantify the antibody response to OAg, as well as to other *Salmonella* antigens such as flagellin, and outer membrane proteins would help understand this better.

This chapter supports previous observations that antibody plays an important role in cell-mediated immunity to NTS (151) and strengthens the rationale for development of a NTS vaccine that accelerates the natural process of antibody acquisition against NTS (50). However, we need to determine if the findings of impaired cell-dependent killing in the presence of high anti-*Salmonella* antibody titres is only an in vitro observation or whether this phenomenon occurs in vivo, in post-vaccinated individuals who have high anti-*Salmonella* antibody titres.

7.5 CONCLUSIONS

NTS bacteraemia is common in young Malawian children. In sera from some children, this antibody can induce cell-mediated killing of *Salmonella* but the high titres of antibody which are produced in response to infection with invasive isolates of NTS may not necessarily be protective. Diluting and supplementing these sera with exogenous complement enhanced killing in these sera, suggesting that absent in vitro killing with undiluted serum could be a result of high levels of anti-*Salmonella* antibody diverting

complement deposition, insufficient complement levels, the presence of inhibitory anti-OAg antibodies or a combination of all of these that only when serum is diluted, the opsonic potential of antibodies present in the serum is revealed. This work supports the development of a vaccine which induces antibody against *Salmonella* for use in Africa.

CHAPTER 8. SENSITIVITY OF MALAWIAN *SALMONELLA* ISOLATES TO KILLING BY ANTIBODY

8.1 INTRODUCTION

In Sub-Saharan Africa, nontyphoidal *Salmonellae* are a major cause of bacterial blood stream infections (18-20;43). Multi-drug resistant (MDR) NTS have recently been reported in many African countries (43;49;223;224) and this makes treatment difficult. In Malawi, 90% of invasive NTS isolates are resistant to ampicillin, chloramphenicol and cotrimoxazole, but are still sensitive to ceftriaxone and ciprofloxacin (21;49;224). However, it is unlikely to be long before resistance to these antibiotics emerges. It is therefore essential that an alternative strategy is developed which provides protection against NTS, such as a vaccine.

Salmonellae are facultative intracellular organisms which are adapted to survive within macrophages (84), but are also capable of extracellular growth in the blood. Consequently, both cell-mediated and humoral immunity are required to control infection when bacteria are residing within phagocytes and are in transit from cell to cell (74). Antibody protects against the uncontrolled spread of NTS through opsonisation and uptake by blood phagocytes (151) and also through activating complement-mediated lysis of the bacteria (50). The sensitivity of Malawian NTS isolates to opsonic and bactericidal antibody therefore needs to be assessed to determine how effective antibody will be in preventing NTS bacteraemia.

8.2 OBJECTIVES

As part of a larger study investigating the sensitivity of Malawian NTS isolates to bactericidal antibody, we examined the sensitivity of a subset of Malawian NTS isolates to opsonic antibody and killing by peripheral blood cells.

8.3 RESULTS

8.3.1 SEROVAR DISTRIBUTION AND CASE FATALITY RATES OF NTS BACTERAEMIA

In this study, 350 NTS isolates were cultured from blood taken from children admitted to the Queen Elizabeth Central Hospital, Blantyre, Malawi, between 1st August 2003 and 31st July 2004. The median age of children with NTS bacteraemia was 13 months and 51% were boys. 91% of the isolates were *Salmonella* Typhimurium, 8% were *Salmonella* Enteritidis. One isolate was *S. Bovismorbificans*, one *S. Sundsvall*, one untypable and seven were identified as *Salmonella* Typhi. 94% of the *Salmonella* Typhimurium isolates were multi-drug resistant (MDR) and 48% of the *S. Enteritidis* were MDR. Case fatality rates were 19.0% for those children infected with *S. Typhimurium* and 22.7% for those infected with *S. Enteritidis*.

8.3.2 SENSITIVITY OF MALAWIAN NTS ISOLATES TO KILLING BY ANTIBODY

In work prior to this thesis, serum bactericidal assays were performed by Dr Chisomo Msefula to screen the NTS isolates and determine if the sensitivity to bactericidal

antibody varied. Serum bactericidal assays were performed on the available 329 isolates (94% of the isolates cultured from bacteraemic children). These assays assess the sensitivity of *Salmonella* to antibody-induced complement-mediated lysis of the bacteria. Each clinical isolate was tested with an aliquot of sera from the same pool of ten healthy HIV-uninfected Malawian adults (pooled Malawian serum).

The majority of the isolates were killed after 180 min exposure to antibody and complement (Figure 8.1). 19% of the clinical isolates were killed by at least 3.0 log₁₀ (1000 fold reduction in bacterial numbers compared to the initial concentration of bacteria) after 180 min and were classified as 'highly susceptible' isolates. 72% of the isolates tested were killed between 1.4 log₁₀ and 3.0 log₁₀ and were classified as 'susceptible'. This 'susceptible' group included *Salmonella* Typhimurium D23580, an isolate which we routinely use in our laboratory as a characteristic, representative Malawian isolate (217). 8% of the isolates were in the range of 0 – 1.4 log₁₀ kill and these were classified as 'moderately resistant'. Only 5 isolates, (1.5% of the total isolates tested), were fully resistant to serum killing and increased in numbers after 180 min. These 5 isolates were classified as 'resistant'.

8.3.3 SENSITIVITY OF MALAWIAN NTS ISOLATES TO OPSONIC ANTIBODY AND KILLING BY PERIPHERAL BLOOD CELLS

Eleven NTS isolates which represented a range of susceptibilities to antibody-induced complement-mediated killing were selected for further investigation. One of the eleven isolates was 'highly susceptible'; three were 'susceptible' (including *S. Typhimurium*

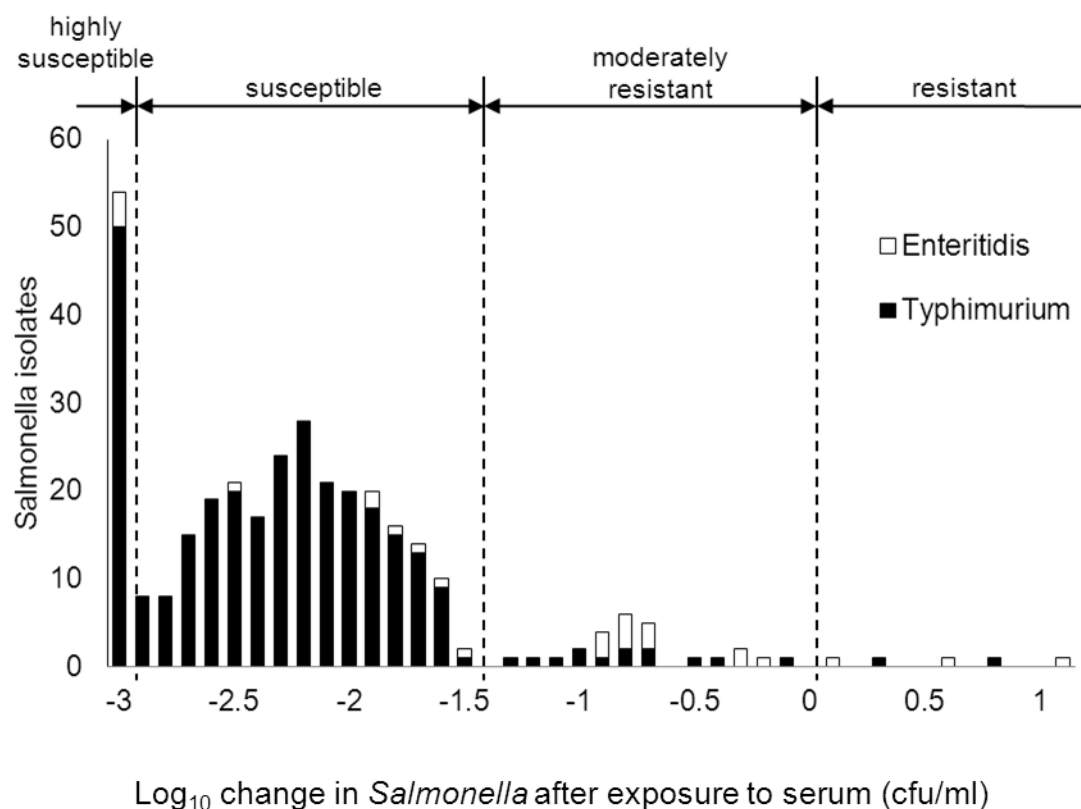


Figure 8.1 Sensitivity of Malawian invasive NTS isolates to bactericidal antibody. Sensitivity of 329 NTS isolates to serum killing at 180 min in the serum bactericidal assay using pooled sera from Malawian adults demonstrating 'highly susceptible', 'susceptible', 'moderately resistant' and 'resistant' groups.

D23580), three 'moderately resistant' and four 'resistant' to killing by the pooled sera in the SBA. Six were *S. Typhimurium* and five were *S. Enteritidis*.

To determine if the susceptibility to opsonic antibody varies for the invasive Malawian NTS isolates, blood cell killing assays were performed with the eleven selected NTS isolates (Figure 8.2 A). The isolates were opsonized with aliquots from the same batch of adult pooled Malawian serum as used for the serum bactericidal assays. The opsonised 'highly susceptible' isolate underwent the greatest kill by peripheral blood cells, with an average kill of $-1.8 \log_{10}$. The 'susceptible' group underwent a -1 to $-1.7 \log_{10}$ kill. The 'resistant' and 'highly resistant' underwent a -0.5 to $-1 \log_{10}$ kill and $-0.5 \log_{10}$ kill to $1.0 \log$ growth respectively. Killing of the eleven NTS isolates tested in the blood cell assay correlated with killing in the serum bactericidal assays (Figure 8.2 B) (Spearman $r_s=0.90$, 95% CI 0.64 to 0.97, $p=0.0002$).

8.3.4 SENSITIVITY OF ISOLATES TO PHAGOCYTOSIS AND OXIDATIVE BURST

The phagocytic response of peripheral washed blood cells to the eleven opsonised isolates was assessed using a flow cytometry-based assay (Figure 8.3 A and B). The isolates were opsonised with pooled Malawian serum or PBS (non-opsonised). The mean percentage phagocytosing neutrophils for the 'highly susceptible'/'susceptible' isolates was 73% (range 65.15 – 79.50) and not significantly different from that (69%

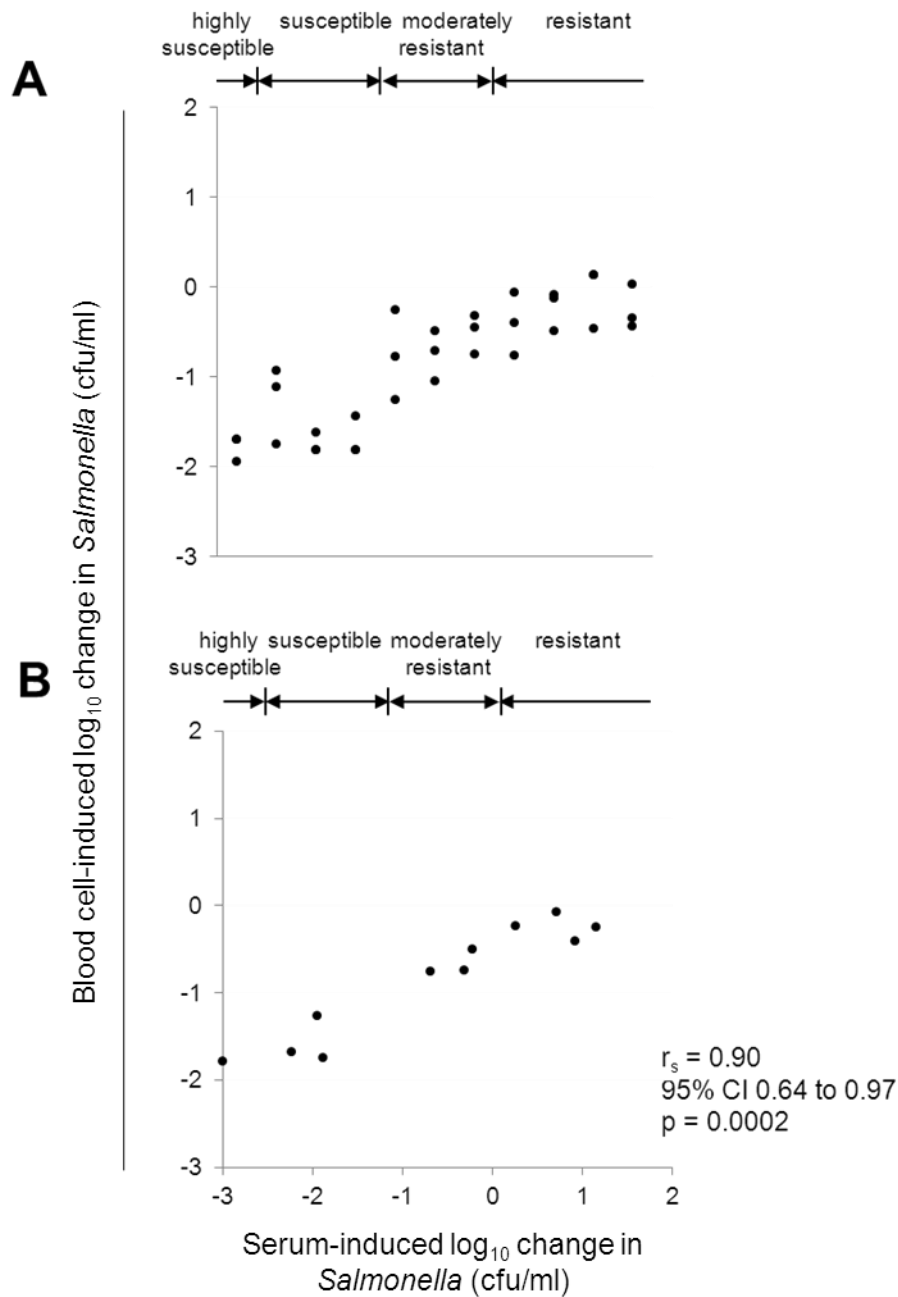


Figure 8.2 Antibody-dependent killing of NTS isolates by washed peripheral blood cells.

(A) Killing of the eleven Malawian NTS isolates at 180 min in the *Salmonella* cell killing assay by washed peripheral blood cells following opsonisation with pooled Malawian sera. Each vertical set of three circles represents data from three independent experiments. (B) Killing of each NTS isolate opsonised by the adult Malawian pooled sera, by blood cells (means of each set of 3 results from A) compared with killing by serum alone (values from Figure 8.1). r_s = Spearman's correlation.

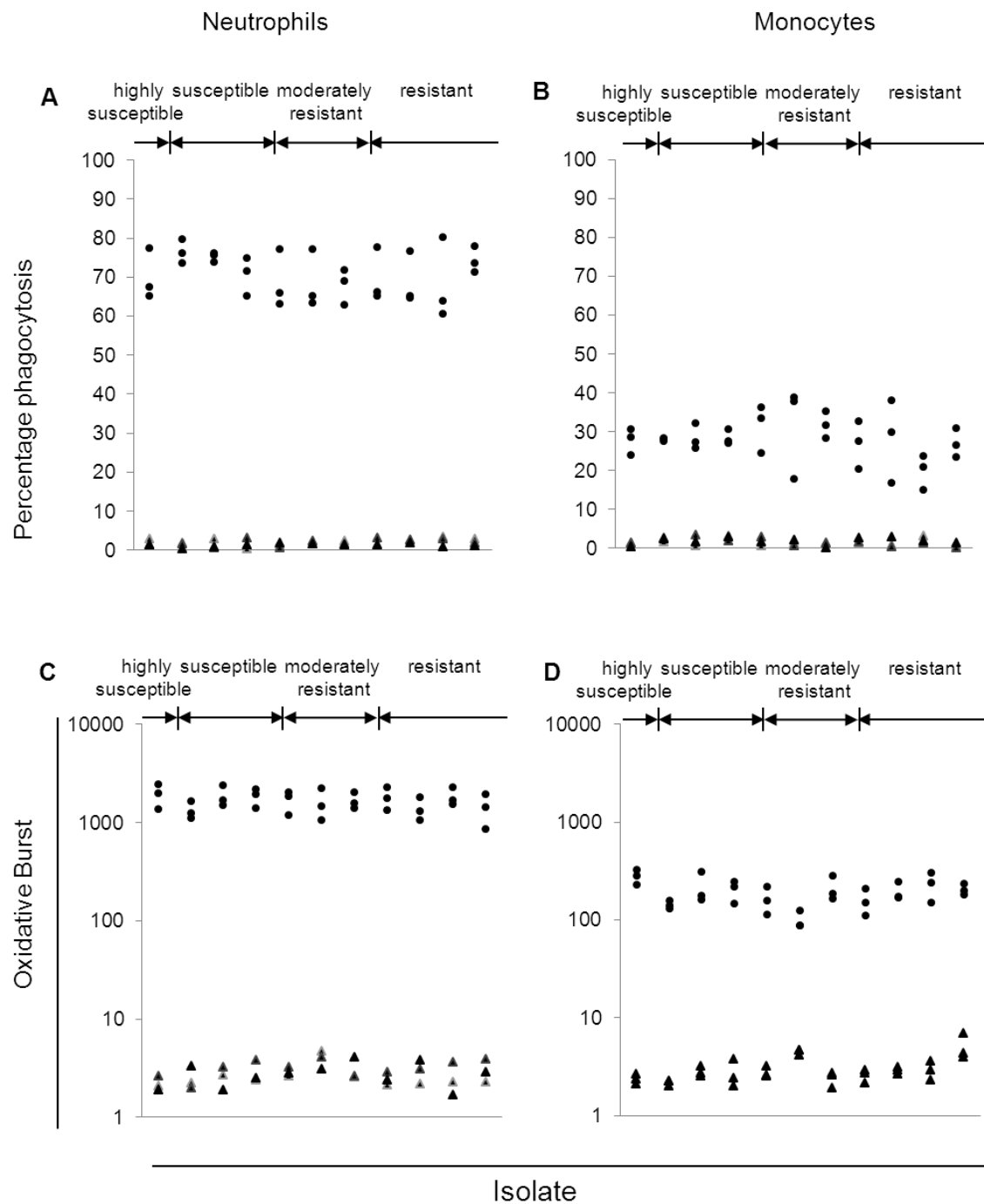


Figure 8.3 Antibody-dependent oxidative burst and phagocytosis by peripheral blood cells following stimulation with invasive Malawian NTS isolates.

Phagocytic response by neutrophils (A) and monocytes (B) and oxidative burst activity by neutrophils (C) and monocytes (D) in washed peripheral adult blood in response to stimulation with each NTS isolate opsonised with pooled Malawian serum (circles) or opsonised with PBS (triangles). Each vertical set of three circles or triangles represents data from three independent experiments with one isolate.

(range 62.92 – 80.07)) for the 'moderately resistant'/'resistant' isolates (student's t-test $p=0.09$) (Figure 8.3 A). The mean percentage phagocytosing monocytes (Figure 8.3 B) for the 'highly susceptible'/'susceptible' isolates and 'moderately resistant'/'resistant' isolates was the same (mean 28%). Opsonisation of the eleven isolates with PBS (non-opsonised) did not lead to phagocytic activity for neutrophils or monocytes (Figure 8.3 A and B).

The ability of the eleven isolates to stimulate oxidative burst activity in neutrophils was similar for both groups (mean oxidative burst response for 'highly susceptible'/'susceptible' isolates was 1729 U (range 1102 U to 2443 U) and 1612 U for 'moderately resistant'/'resistant' isolates (range 857 U to 2276 U)) (Student's t-test $p=0.45$) (Figure 8.3 C). The mean oxidative burst by monocytes (Figure 8.3 D) was not significantly different for 'highly susceptible'/'susceptible' isolates and the 'moderately resistant'/'resistant' isolates (means 207 U and 178 U) (Student's t-test $p=0.34$). Stimulation of neutrophils and monocytes with non-opsonised isolates (Figure 8.3 C and D) did not induce oxidative burst activity.

8.3.5 ANTIBODY AND COMPLEMENT DEPOSITION ON MALAWIAN ISOLATES

Cell-mediated and cell-independent killing of invasive NTS isolates is dependent on both binding of antibody to the bacteria, and complement deposition on the surface of the bacteria. To determine whether variation in susceptibility to bactericidal antibody

observed in the 329 NTS isolates and variation in the susceptibility of blood cell killing of 11 isolates is due to reduced antibody binding to some isolates, flow cytometric anti-*Salmonella* IgG binding assays using the same pooled sera as before were performed on the eleven isolates (Figure 8.4 A). The minimum binding of IgG required to induce complement lysis of *S. Typhimurium* D23580 is 1.5 U (50). Binding of IgG to each of the eleven isolates was greater than 1.5 U.

Complement deposition assays were performed on the same eleven isolates, again using the same batch of pooled Malawian sera and the deposition of C3 and the C5b-9 membrane attack complex was measured by flow cytometry. For C3 deposition (Figure 8.4 B), the median deposition was 3700 U (range 2800 U to 4700 U), which exceeds 100 U - the C3 deposition level previously shown to be required to kill *S. Typhimurium* (50). For C5b-9 (Figure 8.4 C), the median level was 22 U (range 13 U to 36 U), again exceeding the level of 0.6 U which is required to effect bacterial lysis. There was no correlation between C3 or C5b-9 deposition and sensitivity to antibody-mediated killing for each isolate (Spearman $r_s = -0.35$, 95% CI 0.28 to -0.77, $p=0.27$) and (Spearman $r_s = 0.30$, 95% CI 0.74 to 0.34, $p=0.35$) respectively.

The results of these assays suggest that the difference in sensitivity to antibody-dependent complement-mediated killing of various NTS isolates do not relate to the amount of anti-*Salmonella* IgG antibody or complement components binding to the surface of the isolate.

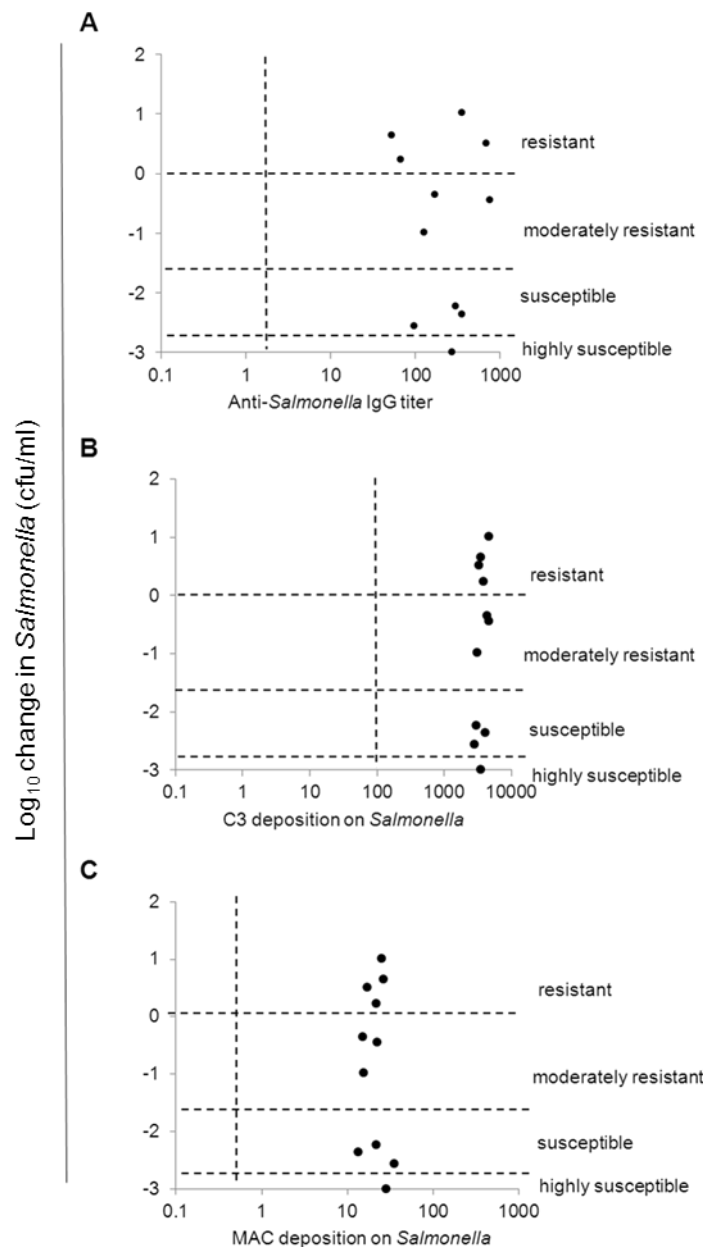


Figure 8.4 Antibody and complement deposition on eleven Malawian NTS isolates. (A) Titre of IgG antibody in pooled Malawian adult sera binding to 11 isolates (selected from the isolates tested in Figure 8.2, which represent a range of sensitivities to antibody-mediated killing) plotted against SBA result at 180 min for each isolate. (B) C3 and (C) C5b-9 membrane attack complex from pooled Malawian sera on each NTS isolate. Vertical dashed lines represent minimum thresholds of IgG binding and complement deposition required to kill *S. Typhimurium* D23580. There was no correlation between C3 or C5b-9 deposition and sensitivity to antibody-mediated killing for each isolate ($r_s = -0.35$, 95% CI 0.28 to -0.77, $p=0.27$) and ($r_s = 0.30$, 95% CI 0.74 to 0.34, $p=0.35$) respectively.

8.4 DISCUSSION

This chapter re-emphasizes that NTS is a major cause of bacteraemia amongst children in Malawi. Within a 12 month period, there were 350 cases of NTS bacteraemia in children admitted to one hospital in Blantyre, Malawi. In keeping with other observations in African countries, infection with *Salmonella* Typhimurium was more common than *Salmonella* Enteritidis (50;225). 89% of the isolates were also multi-drug resistant highlighting the need for a vaccine for use in African countries.

In order to develop effective vaccines against invasive NTS diseases, the relative sensitivity of common invasive isolates to antibody needs to be established, and this was the main aim of this study. The susceptibility to antibody-dependent complement-mediated killing of 329 isolates from bacteraemic children, and sensitivity to opsonic antibody of eleven isolates was assessed using a pool of serum from 10 healthy, HIV-uninfected Malawian adults. The same batch of pooled sera was used throughout and provided a broad range of antibody specificities. Healthy adults from Blantyre were chosen as antibody to NTS is acquired during childhood, and these adults are likely to have been exposed to similar NTS isolates used in this study. The serum bactericidal assay used in this study assesses bactericidal activity in 100% human serum and is more likely to reflect killing in vivo than some bactericidal assays which use diluted animal serum. Likewise for the cell killing assays, fresh human peripheral blood cells from healthy subjects were used rather than cultured phagocytic cell lines. Cell lines are technically difficult to maintain and may not express the appropriate FcR phenotype to

mimic human phagocytes in vivo. Through the use of human serum as opsonins and human blood phagocytes, the assays help to match physiological conditions.

The susceptibility of the NTS isolates to antibody-dependent complement-mediated killing varied. The majority (74%) of the NTS isolates were 'moderately susceptible', 16% were 'highly susceptible', less than 10% were 'moderately resistant' and 1.5% were 'resistant' to killing via this mechanism. A range of susceptibility to killing of opsonised *Salmonella* by peripheral washed blood cells was observed for our collection of isolates. Surprisingly, phagocytosis by neutrophils and monocytes and the oxidative burst response, measured by flow cytometry, was similar for all isolates. The batch to batch variation in the efficiency of fluorescent-labelling the NTS isolates can be a major variable in the opsonophagocytosis assay and may contribute to this finding. Shaio *et al* monitored the phagocytic index for six strains of *Salmonella* Typhimurium, and found that at high serum concentrations, the phagocytic index was hardly discernible between the isolates, but at 1% serum, differences were observed (226). Therefore, we may have observed optimal uptake for all eleven isolates as there was excess antibody and complement present and any differences in sensitivity to opsonic antibody would not be detected.

To try to account for the differences in susceptibility to antibody we examined the binding of antibody and complement to the isolates. The binding of IgG and deposition of C3 and C5b-9 on each of the isolates far exceeded the thresholds needed to drive killing of the bacteria, suggesting that the lack of killing for some isolates is not due to a

lack of antibody or complement binding. Joiner *et al* showed that for *Salmonella* Montevideo, the C5b-9 membrane attack complex forms but fails to be inserted into the cell membrane thus impairing cell lysis (186). It is possible that we are detecting MAC formation in our assay but that the MAC is formed too far away for insertion into the membrane or that it falls off.

Interestingly, susceptibility to antibody-mediated cell-dependent killing correlated with susceptibility to cell-free lysis of the bacteria. This suggests that opsonic antibody may not be able to compensate for the lack of antibody-dependent cell-independent killing of 'resistant' NTS isolates as previously postulated (151). 'Resistant' NTS isolates may have inherent mechanisms that confer general broad resistance to killing by immune effectors. This concept is supported by the finding that resistance to both modalities of killing occurs despite no obvious reduction in antibody-binding, complement deposition, phagocytosis of the bacteria or stimulation of the oxidative burst function. The observed large group of susceptible isolates could reflect the high prevalence of infection with *Salmonella* Typhimurium D23580-related isolates during this period in Malawi, which have this level of sensitivity to antibody or the dominance of these isolates may be a consequence of their relative susceptibility to antibody-mediated killing.

It is unclear why invasive disease caused by NTS that are highly susceptible to antibody killing occurs. It is possible that children infected with these NTS isolates don't have *Salmonella*-specific antibody, but the mean age of infection for children infected by this

group of isolates was not lower than the ages of other groups. It is also possible that these isolates adopt a more serum-resistant phenotype in vivo, or that the infected children have an underlying susceptibility to *Salmonella* infection.

8.5 CONCLUSIONS

In conclusion, some NTS isolates from bacteraemic children in Malawi were found to be sensitive to opsonic and bactericidal antibody, suggesting that an antibody-inducing vaccine might successfully protect against the majority of invasive NTS isolates, particularly in communities where multi-drug resistant isolates are common. Some isolates were resistant to both opsonic and bactericidal antibody and therefore a vaccine which induces T-cell immunity as well as antibody would be optimal as it minimises the risk of strain replacement with antibody-resistant isolates.

CHAPTER 9.

GENERAL DISCUSSION

NTS are a major cause of invasive disease in Sub-Saharan Africa (18;21;199), with mortality rates as high as 24% in children with NTS bacteraemia (18) and 45% for HIV-infected adults (30). With the emergence of multidrug resistance (21), a vaccine which provides protection against NTS is urgently needed. In order to develop a vaccine for use in Africa, a further understanding of the immune response to NTS is required. It has previously been shown that antibody plays an important role in immunity to NTS in African children and adults (50;61;151) and the aim of this PhD was to further understand the role of opsonic antibody in cell-mediated immunity to NTS.

The availability of sera from children with NTS bacteraemia, and adults infected with HIV from field studies in Malawi, provided a useful resource for studying cell-mediated immunity in the most affected populations who are at greatest need of a vaccine. The preliminary project which defined the optimal guidelines for handling serum minimised the waste of such limited African sera. The findings that serum could undergo three freeze-thaw cycles and be stored at 4°C for extended periods of time, or at 22°C for 9 days was surprising given that complement is known to be labile and the accepted view that freeze-thaw cycles should be avoided. The observation of no detrimental effect on serum bactericidal activity following delayed separation of serum from clotted blood stored at 4°C or 22°C for up to four days post-venesection, has diagnostic and clinical implications as well as important implications for resource poor settings, such as clinics

in Africa, where equipment and storage facilities such as -80°C freezers may not be available.

Another aim of this PhD was to optimise our existing in vitro antibody cell-mediated killing assay to ascertain the minimal titres of serum that can still drive cell-mediated killing. Although commonly used for opsonophagocytic assays for other bacteria (192;196), baby rabbit serum was found not to be a suitable exogenous complement source as it was toxic to *Salmonellae* at high concentrations, and could not induce blood cell killing of *Salmonellae* when used at low concentrations together with anti-*Salmonella* antibody. Human antibody-depleted serum was found to be a suitable complement source for use in our blood cell killing assay and it keeps assay conditions more closely related to those in vivo in man. The complement and antibody requirements for this assay were compared with those for cell-free antibody-dependent complement-mediated killing of NTS. Antibody and complement thresholds for cell-free killing of *Salmonella* were much lower than those required for cell-killing. By identifying antibody-depleted human serum as a suitable complement source and the percentage of complement and antibody required to opsonise *Salmonella* and induce killing by peripheral blood cells in our blood cell killing assay, we have identified an assay which can be used to measure titres of natural and vaccine-induced opsonic antibody for killing of NTS.

NTS infections are commonly associated with HIV infection (30) and we studied the role of antibody in cell-mediated immunity in HIV-infected Malawian adults to try to better

understand the cause of this association. Impaired phagocytosis and killing of *Salmonella* opsonised with sera from some HIV-infected Malawian adults by washed peripheral blood cells was observed. It has long been known that a factor within serum can block bactericidal activity of serum against common gram negative bacilli (227). We previously identified that high titres of anti-OAg antibodies found in serum from HIV-infected African adults inhibits bactericidal killing of *Salmonella* (61). Through the development of an affinity chromatography method to purify anti-OAg antibodies, we were able to characterise the role of anti-OAg antibodies in cell-mediated immunity to NTS. We determined that high titres of anti-OAg antibodies found within the serum from some HIV-infected African adults inhibit blood cell killing of *Salmonella* in a concentration-dependent manner, with inhibition diminished as the concentration of anti-OAg antibodies was decreased. Further work is required to determine the precise mechanism of OAg-antibody related inhibition of cell-mediated killing. As has been proposed for cell-free, antibody-dependent complement-mediated immunity (61), excess anti-OAg antibodies could be blocking access of other antibodies to targets on the membrane and/or diverting complement access. Further investigation is required to identify more fully the inhibition mechanism(s). Alternatively, anti-OAg IgA antibodies could interfere with C3 fixation or block the binding of IgG or hinder the binding of IgG to the FcR. Purification of these anti-OAg IgA antibodies from the sera would be useful. Furthermore, biacore analysis of the binding of purified anti-OAg antibodies from HIV-infected inhibitory and non-inhibitory sera to the OAg would give an insight into whether the affinities of the antibodies for the OAg are different and whether this could explain the observed inhibition. Overall, this study contributes

another explanation for the high incidence and mortality observed in Africa for HIV-infected adults with NTS bacteraemia, as it appears that both cell-mediated and cell-free (61) mechanisms are impaired in these individuals.

We also studied the role of opsonic antibody in immunity to NTS in children with bacteraemia. The majority of these children were under 2 years of age which highlights the incidence of NTS bacteraemia in young children and the need for a vaccine which accelerates protective natural antibody acquisition for this population. This work also supports the importance of antibody in cell-mediated immunity to NTS, as for the majority of the children's sera, antibody acquired during infection effectively opsonised *Salmonella* and triggered killing by washed blood cells suggesting they can provide protection against future infection. Anti-*Salmonella* antibody titres increased post-infection but high titres of antibodies were not necessarily protective, with blood cells failing to kill *Salmonella* opsonised with some sera. However, when diluted, the majority of sera induced blood cell killing suggesting the presence of an in vitro prozone effect (222) possibly caused by limited complement, or excess anti-*Salmonella* or anti-OAg antibodies blocking or diverting complement deposition. It is possible that this is an in vitro artefact of the assay and would not happen in vivo in vaccinated individuals. Further work is required to understand why a subset of children's sera that contain high titres of anti-*Salmonella* antibody cannot kill *S. Typhimurium*. Whether high OAg antibody titres impair cell-mediated killing of *Salmonella* in vivo in this subset of infected individuals needs further investigation. Although none of the children in the

study were infected with *S. Typhimurium* D23580, almost all of the children could induce blood-cell killing of *S. Typhimurium* D23580 suggesting that antibody induced by infection with one NTS isolate is cross-protective and can be opsonic against other Malawian isolates of NTS. This could also be explained by the finding that *S. Typhimurium* isolates in Malawi since 2002 are all from the same clade and are part of ST313 (217).

A study on the sensitivity of Malawian NTS isolates to antibody-mediated killing confirmed that some NTS isolates found in the community in Blantyre, Malawi are sensitive to bactericidal antibody, and some isolates are also sensitive to opsonic antibody and cell-mediated killing. Resistance to cell-mediated immunity correlated with resistance to bactericidal killing for 11 isolates ranging in their level of serum resistance, suggesting that a successful NTS vaccine needs to induce both opsonic and bactericidal antibody as well as T-cell mediated immunity. Larger studies are required in other countries in Sub-Saharan Africa that are affected by the NTS epidemic, to determine the relative sensitivity of isolates from these areas to antibody, and the overall potential coverage of an antibody-inducing vaccine.

The work undertaken during this PhD reinforces the important role antibody plays in immunity against NTS and supports the development of a vaccine which induces the production of antibody to *Salmonella*. In order to do this, the targets of protective opsonic and bactericidal antibodies need to be established and this should be the focus of future work. Given the evidence of protective immunity when prototype OAg

conjugate vaccines have been tested in rabbits and mice (156-158), the development of polysaccharide-conjugate vaccines for use in humans is a promising avenue, and a conjugate vaccine based on the OAg of *Salmonella* is currently under development at NVGH. Work carried out within this PhD suggests that anti-OAg antibodies can be opsonic when present at the serum titre found in healthy Malawian adults, but at the serum titres of some HIV-infected adults, they inhibit both cell-dependent and cell-independent (61) immunity. This cautions against the use of OAg as a vaccine candidate. Furthermore, an OAg-based vaccine has the problem of serovar-specificity and consequently strain replacement is a concern. Given that not all invasive Malawian isolates of NTS are sensitive to opsonic and bactericidal antibody, this could be an issue. Therefore, the common target antigens of protective responses to NTS need to be identified. Ideally, the vaccine target should induce opsonic and bactericidal antibodies. Antibody targeting the outer membrane of *Salmonella*, in particular ompD (182), have been shown to be bactericidal (61) and further studies are required to understand their role in opsonisation. A method to purify antibodies to the outer membrane proteins from human serum also needs to be developed to enable, as for OAg antibodies, the characterisation of their role in cell-mediated immunity to NTS.

CHAPTER 10.

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APPENDIX A

APPENDIX B
